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## In what Form does Histamine Occur in Plasma?

By

H. COLLEDAHL, CARL G. HOLMBERG and O.-B. LAURELL.

Received 8 March 1946.

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BARSOU and GADDUM (1935) showed that histamine occurs in the blood of mammals. This has been confirmed by several later investigators. Recently EMMELIN (1945) made quantitative analyses of plasma from different animals. He found a very high histamine content in guinea-pig plasma — about 200  $\gamma$ /l — while the histamine content in plasma from all other animals investigated rarely exceeded 100  $\gamma$ /l.

Most investigators think that plasma histamine is present in a bound, inactive form, but EMMELIN has made some experiments from which he infers, that it must be free and active. As it seems important to know in what state the plasma histamine really exists *in vivo*, we have made some investigations aiming at solving this question.

1) If a certain quantity of histamine, for instance 0.5  $\gamma$ , is injected intravenously during 3—5 sec. into an atropinized cat under chloralose a reproducible fall in blood pressure is obtained. We tried to ascertain whether this fall in blood pressure depends on the concentration of histamine in the injection fluid. We found that this was not the case. In the concentration range from 1000  $\gamma$ /l to 25  $\gamma$ /l the effect of 0.5  $\gamma$  histamine is always the same. As the lowest concentration is identical with or lower than the concentration of histamine in cat plasma, it seems impossible that the histamine in cat plasma should be present in an active form.

In some cases our dilutions have been prepared with a solution of dextran of the same colloid osmotic pressure as plasma in order to prevent a possible escape of water from the blood stream. Controls have shown this precaution to be effective.

2) In the experiments of EMMELIN heparin or chlorazol fast pink have been used as anticoagulants. ROCHA E SILVA et al. (1941 and 1943) have shown, that histamine occurs in the cells

in a peptide linkage which can be split by trypsin. It is also known that thrombokinase can be replaced by trypsin as an activator of prothrombin. As heparin and probably also chlorazol act as antithrombins there is a possibility that plasma histamine, if the binding of the plasma histamine is similar to that of the intracellular histamine, might be set free during the preparation, possibly through the action of thrombokinase. In order to find out if this were possible, we have performed some experiments of the following type: Blood was drawn by heart puncture from a guinea-pig and to one part heparin was added (0.125 mg/ml.), to another citrate (5.4 mg/ml.). The blood corpuscles were centrifuged down and equivalent parts of heparinized and citrated plasma respectively were injected intravenously into a chloralozed, atropinized cat. The result was a fall in blood pressure after heparin plasma of the order of magnitude which might be expected if the plasma had a histamine content of about 200  $\gamma$ /l; citrate plasma, on the other hand, had no effect whatsoever on the blood pressure. Controls show that citrate in the concentration used has no effect on the fall in blood pressure produced by histamine. The time from heart puncture to test was 1—2 hours.<sup>1</sup>

In our opinion these experiments strongly support the hypothesis that histamine is present in plasma in a bound inactive form, and that it might be set free already during the sampling of plasma, possibly through the action of thrombokinase. We are at present investigating the possible rôle, played by the bound plasma histamine in the coagulation mechanism and in certain anaphylactic reactions.

### Summary.

Two types of experiments have been performed, both of which indicate that histamine is present in plasma in bound, inactive form.

The possibility is discussed that histamine might be set free during the sampling of blood through the action of thrombokinase.

### Literature.

- BARSOUM, G. S., and J. H. GADDUM, *J. Physiol.* 1935. 85. 1.  
DRAGSTEDT, C. A., and M. ROCHA E SILVA, *Proc. Soc. Exp. Biol. Med.*, 1941. 47. 420.  
EMMELIN, N., *Acta Physiol. Scand.* 1945. 11. Suppl. 34.  
ROCHA E SILVA, M., and S. O. ANDRADE, *J. Biol. Chem.* 1943. 149. 9.

<sup>1</sup> Experiments have also been performed with ultrafiltrates from heparinized and citrated plasmas respectively. The results are almost quantitatively the same as with plasma, i. e. the same drop in blood pressure in the first case but none in the second.

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## Study on Ph. Broemser's Manometer Theory for Oscillations in the Aorta.

By

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### Theoretical Part.

Frank-Broemser's manometer theory for oscillations in the aorta is based on an analogy which makes it possible to treat these oscillations mathematically in the same way as a damped linear oscillator is treated in mechanics. The aorta is an elastic tube ("manometer"). At a point, near the heart, acts a pressure (the central pulse pressure) which is periodical with the time period  $T$  = the pulse time. This pressure can be mathematically expressed in a Fourier series.

$$1) \quad P = A_0 + \sum_{n=1}^{\infty} A_n \cos (n\omega t - \varphi_n),$$

where  $\omega = \frac{2\pi}{T}$  and  $t$  = the time in seconds.

At a point on the aorta lying near its lower end, the likewise periodical, peripheral pulse pressure is measured.

$$2) \quad P' = A'_0 + \sum_{n=1}^{\infty} A'_n \cos (n\omega t - \varphi'_n).$$



The tube acts as an elastic system with a distributed mass, which transmits the variable pressure  $p$  to the pressure point  $p'$ . These pressures give rise to oscillations in the walls of the vessels. The oscillations are damped.

This moving system is conceived, from a mechanical point of view, to be equivalent to the following: An elastic spring is provided at one end with a mass point  $m$ . The other end is forced to swing in a periodic motion, so that its shifting  $z$  from the middle position

is a periodic function with the time period  $T$ . Owing to this oscillatory movement, the mass point will be forced into a motion in which its deviation from the middle position is equal to  $x(t)$ . The mass of the spring can be reduced to an equivalent mass, which is included in the mass  $m$ . The damping force is assumed to be proportional to the velocity of the mass point, and is conceived to be likewise reduced to a concentrated force, acting on the mass point. For  $x(t)$  we then obtain an equation:

$$3) \quad \frac{d^2x}{dt^2} + 2D \frac{dx}{dt} + q^2x = q^2z,$$

where  $D$  and  $q^2$  are positive constants.

If  $z$  in the result and  $x$  are assumed to be proportional to the pressures  $p$  and  $p'$ , respectively, and if the proportionality constants are equal, we can also write:

$$4) \quad \frac{d^2p'}{dt^2} + 2D \frac{dp'}{dt} + q^2 \cdot p' = q^2 \cdot p.$$

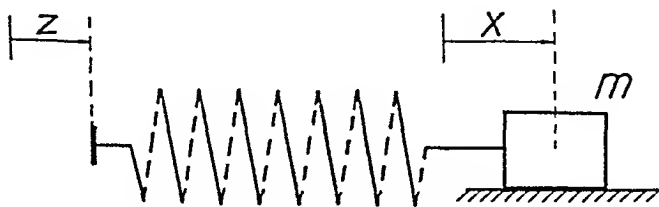


Fig. 2. A mechanical analogy to BROEMSEN's manometer theory.

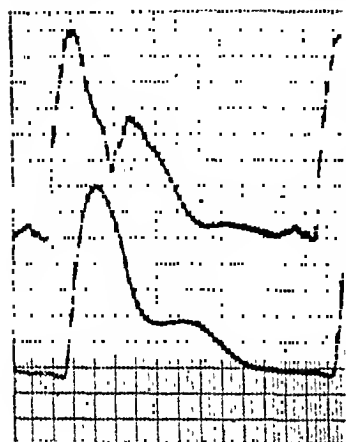


Fig. 1. Pulse curve from a healthy man, aged 37 years. The ordinate: pressure variations. The abscissa: the time graduated in 0.02 seconds. The upper curve: art. subclavia sin. The lower curve: art. iliaca sin.

If  $p$  has the form 1), and if we include merely the variable terms, which are of interest here, we obtain for  $p'$  a solution of the form

$$5) \quad p' = \sum_{n=1}^{\infty} \lambda_n A_n \cos (n\omega t - \varphi_n - \psi_n),$$

where the "magnification factors"  $\lambda_n$  and the phase displacement angles  $\varphi_n$  are determined by the formulas:

$$6) \quad \lambda_n = \frac{1}{\sqrt{(1 - R_n^2)^2 + 4D^2 R_n^2}},$$

where

$$7) \quad R_n = \frac{n\omega}{\underline{q}} = n \cdot R, \quad R \text{ being equal to } \frac{\omega}{\underline{q}}.$$

( $R$  = the manometer's self-oscillation time with undamped oscillation, expressed in the pulse time as a unit of time).

$$8) \quad \operatorname{tg} \psi_n = \frac{2DR_n}{1 - R_n^2}$$

the angle  $\psi_n$  being taken within the interval  $0 < \psi_n < \pi$ .

By comparison with formula 2), we obtain:

$$9) \quad \begin{cases} A'_n = \lambda_n A_n \\ \varphi'_n = \varphi_n + \psi_n \end{cases}$$

The amplitudes  $A_n$ ,  $A'_n$  and the phase angles  $\varphi_n$ ,  $\varphi'_n$  can be directly computed by Fourier analysis of pressure curves taken at suitable places on the aorta.

We shall show here how, from these results, it is possible in several different ways, to compute the magnitude  $R = \frac{\omega}{\underline{q}}$  and thus also  $D$ , which should be constants. If this is really the case, it should bear out Broemser's theory.

If  $n$  and  $n_1$  are two integers, we obtain from 9)

$$\frac{A'_n}{A_n} \cdot \frac{A_{n_1}}{A'_{n_1}} = \frac{\lambda_n}{\lambda_{n_1}}.$$

Put

$$10) \quad H_n = \frac{A'_n}{A_n}$$

we thus obtain

$$11) \quad \frac{\lambda_n}{\lambda_{n_1}} = \frac{H_n}{H_{n_1}}.$$

We can also write:

$$\lambda_n = \frac{\cos \psi_n}{1 - R_n^2} = \frac{\cos \psi_{n_1}}{1 - n^2 R^2}$$

whence, for 11), we can substitute:

$$\frac{H_n}{H_{n_1}} = \frac{\cos \psi_n}{\cos \psi_{n_1}} \cdot \frac{1 - n_1^2 R^2}{1 - n^2 R^2}$$

from which we can solve:

$$A) \quad R^2 = \left( \frac{\omega}{q} \right)^2 = \frac{\frac{H_{n_1}}{\cos \psi_{n_1}} - \frac{H_n}{\cos \psi_n}}{n_1^2 \frac{H_{n_1}}{\cos \psi_{n_1}} - n^2 \frac{H_n}{\cos \psi_n}}.$$

Here  $H_n$ ,  $H_{n_1}$ ,  $\psi_n$ ,  $\psi_{n_1}$  are magnitudes which can be directly obtained from the pressure curves. The value of  $R^2$  should be independent of the combination selected for the figures  $n$  and  $n_1$ .

A further possibility of computing  $R^2$  is given by formula 8):

$$\operatorname{tg} \psi_n = \frac{2DR_n}{1 - R_n^2} = \frac{2DnR}{1 - n^2 R^2}$$

from which we obtain

$$\frac{\operatorname{tg} \psi_n}{\operatorname{tg} \psi_{n_1}} = \frac{n}{n_1} \frac{1 - n_1^2 R^2}{1 - n^2 R^2}$$

and, solving here  $R^2$ , we find:

$$B) \quad R^2 = \frac{\frac{1}{n} \cdot \operatorname{tg} \psi_n - \frac{1}{n_1} \cdot \operatorname{tg} \psi_{n_1}}{n \cdot \operatorname{tg} \psi_n - n_1 \operatorname{tg} \psi_{n_1}}.$$

It is noteworthy that this formula (which is not given by Broemser) contains only the phase displacement angles, but not the amplitudes.

It is easily seen that, if the formulas A) and B) are to give the same value for  $R^2$  irrespective of the combinations ( $n$ ,  $n_1$ ), then

$$C) \quad \frac{\sin \psi_n}{n \cdot H_n} \text{ must be } = \frac{\sin \psi_{n_1}}{n_1 H_{n_1}}$$

i. e. the expression  $\frac{\sin \psi_n}{n H_n}$  must be independent of the index number  $n$ .

## Experimental Part.

In order to study  $R$  and  $D$  more closely, we took synchronous pulse curves from the subclavian and external iliac arteries (the femoral artery) with a piezoelectric technique by which it is possible to give the frequency from 0.5 to 100 c/s (and probably over it) with considerable accuracy. The apparatus (designed by Dr. RUNE ELMQVIST) will be described in another publication.

The persons whose pulse curves were thus recorded were healthy subjects who had lain in a horizontal, dorsal position on an observation table. The recording of the curves commenced after the subjects had rested for at least 15 minutes. The curves were afterwards subjected to Fourier analysis.

On the basis of the results obtained in that analysis,  $R$  was computed according to formula  $A$  for 18 normal cases aged 20—40 years. The computation of  $R$  was based on the values  $n$ ,  $n_1 = 1, 2, 3$ , i. e. on the first, second and third harmonics.

The results of these computations are given in Table 1. As regards most of the subjects examined, two periods were recorded; in one case merely a single period and in a few cases three periods.

Table 1.

No.	Age	$R(1, 2)$	$R(1, 3)$	$R(2, 3)$	$R$ mean value	$R$ in seconds
1 . . .	21	0.547	0.435	—	0.491	0.432
2 . . .	21	0.709	0.725	0.672	0.702	0.463
3 . . .	21	0.475	0.886	0.438	0.433	0.494
4 . . .	22	0.520	0.410	0.567	0.499	0.379
5 . . .	25	0.538	0.500	0.666	0.568	0.534
6 . . .	26	0.544	0.525	0.605	0.558	0.603
7 . . .	29	0.540	0.481	0.585	0.535	0.460
8 . . .	30	0.595	0.560	0.640	0.598	0.478
9 . . .	32	0.480	0.392	0.446	0.439	0.457
10 . . .	32	0.501	0.406	0.531	0.480	0.446
11 . . .	34	0.496	0.370	0.451	0.439	0.426
12 . . .	34	0.534	0.350	0.355	0.413	0.330
13 . . .	35	0.530	0.371	0.527	0.476	0.381
14 . . .	35	0.437	0.408	0.421	0.422	0.414
15 . . .	37	0.461	0.408	0.440	0.436	0.523
16 . . .	38	0.609	0.505	0.584	0.566	0.458
17 . . .	38	0.369	0.516	0.335	0.407	0.399
18 . . .	39	0.478	0.337	0.405	0.407	0.379

The table shows quite clearly that the values of  $R$  are independent of the combination ( $n$ ,  $n_1$ ). Furthermore, the condition laid down under C) is controlled as regards the first three harmonics

by forming the expressions  $\frac{\sin \psi_n}{n H_n}$  for  $n = 1, 2$  and  $3$ . The result is given in Table 2. We find that, though the studied expressions are of the same order of magnitude, the variation between the three values from the same curve is so marked that Broemser's theory of linear damping must be taken with reservation.

Table 2.

No.	$\frac{\sin \psi_1}{H_1}$	$\frac{\sin \psi_2}{2 H_2}$	$\frac{\sin \psi_3}{3 H_3}$	No.	$\frac{\sin \psi_1}{H_1}$	$\frac{\sin \psi_2}{2 H_2}$	$\frac{\sin \psi_3}{3 H_3}$
1. . I	0.36	0.42	0.35	11. . I	0.29	0.31	0.52
II	0.31	0.43	0.37	II	0.44	0.39	0.68
2. . I	0.54	0.64	0.04	III	0.34	0.35	0.55
II	0.62	0.28	0.14	12. . I	0.36	0.45	0.57
3. . I	0.24	0.23	0.37	II	0.51	0.36	0.63
4. . I	0.42	0.55	0.54	13. . I	0.36	0.42	0.57
II	0.43	0.60	0.48	II	0.30	0.36	0.43
5. . I	0.36	0.66	0.51	III	0.29	0.25	0.47
II	0.82	0.53	0.29	14. . I	0.41	0.18	0.34
6. . I	0.28	0.51	0.65	II	0.51	0.15	0.28
II	0.23	0.51	0.60	15. . I	0.37	0.23	0.35
7. . I	0.28	0.47	0.29	II	0.38	0.24	0.31
II	0.16	0.50	0.48	16. . I	0.43	0.66	0.44
8. . I	0.56	0.65	0.25	II	0.53	0.70	0.53
II	0.58	0.49	0.35	17. . I	0.43	0.43	0.53
9. . I	0.31	0.31	0.35	II	0.44	0.42	0.38
II	0.32	0.30	0.35	18. . I	0.17	0.34	0.47
10. . I	0.26	0.37	0.49	II	0.19	0.39	0.52
II	0.44	0.36	0.47	III	0.26	0.41	0.59
III	0.22	0.45	0.53				

According to formula (8),  $D$  can be computed if  $R$  is known:

$$D = \frac{1}{2} \left[ \frac{1}{nR} - nR \right] \cdot \operatorname{tg} \psi_n.$$

In the above computations three values for  $R$  have been obtained for each studied curve. By means of these three values, three values of  $D$  can now be obtained for each  $n$  and, by putting  $n = 1, 2, 3$ , altogether nine values for the damping constant can be obtained in respect of each curve. If BROEMSER's theory regarding the nature of the damping were acceptable, these nine values, within the limits of experimental errors, should agree. The calculations indicated here were made for 9 of the studied cases, corresponding to 16 recorded pulse curves, and the results are given in Table 3. These results show distinctly that, in the

Table 3.

No.	n	P computed with		
		R (1,2)	R (1,3)	R (2,3)
1 I . . . . .	1	0.25	0.19	—
	2	0.26	0	—
	3	0.73	0.23	—
1 II . . . . .	1	0.25	0.37	—
	2	0.27	0	—
	3	0.73	0.39	—
2 I . . . . .	1	0.42	0.39	0.48
	2	0.47	0.51	0.55
	3	0.69	0.72	0.72
2 II . . . . .	1	0.45	0.41	0.49
	2	0.49	0.48	0.47
	3	0.40	0.16	0.62
3 I . . . . .	1	0.15	0.26	0.27
	2	0.25	1.49	0.67
	3	0.62	0.38	0.59
4 I . . . . .	1	0.58	0.62	0.59
	2	—	—	—
	3	—	≈ 0.6	≈ 0.57 ≈ 0.51
4 II . . . . .	1	0.56	0.37	0.27
	2	0.43	—	—
	3	0.37	0.62	1.56
5 I . . . . .	1	0.25	0.25	0.25
	2	0.40	0	1.39
	3	0.77	0.51	1.17
5 II . . . . .	1	0.47	0.29	—
	2	0.67	0.20	—
	3	0.39	0.76	—
6 I . . . . .	1	0.24	0.26	0.21
	2	0.40	0.77	0.62
	3	0.38	0.24	0.87
6 II . . . . .	1	0.20	0.27	0.12
	2	0.47	≈ 0	1.56
	3	1.14	0.28	1.54
7 I . . . . .	1	0.20	0.26	0.15
	2	0.38	0.64	0.52
	3	0.34	0.26	0.43
7 II . . . . .	1	0.15	0.16	0.12
	2	≈ 0.34	≈ 0	≈ 0.48
	3	0.62	0.45	≈ 0.52 ≈ 90°
8 I . . . . .	1	0.55	0.51	0.44
	2	0.56	0.63	0.84
	3	0.30	0.31	0.35
8 II . . . . .	1	0.44	0.57	—
	2	0.47	0.11	—
	3	0.60	0.37	—
9 I . . . . .	1	0.24	0.30	0.26
	2	0.18	1.14	0.54
	3	0.70	0.30	0.56

studied oscillations, we *cannot* reckon with the simple damping law suggested by BROEMSER.

### Summary.

Frank-Broemser's manometer theory regarding oscillations in the aorta has been studied, and the following results have been obtained.

1) "The fundamental (self) oscillation time" of the arterial pulse, as computed for 18 healthy subjects, aged 20—40 years, with different methods, gives the same values for each subject, regardless of the combinations of computation.

2) BROEMSER's supposition that the "manometer" has a constant damping factor is not correct.

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## **A Simple Apparatus for Simultaneous Measurement of the Standard Metabolism and the Respiratory Quotient in Small Laboratory Animals, Supplemented with Investigations on Normal and Hypophysectomised Rats.**

By

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One of the simplest and most widely adopted methods for determining the RQ of small laboratory animals is no doubt the gravimetric method described by HALDANE in 1892. MITCHELL and CARMEN (1926) have described a device for using HALDANE's apparatus both for determining the RQ and the standard metabolism. A great drawback of this arrangement is that it is absolutely necessary to observe the animals in the experimental period.

I have therefore tried to modify KROGH's apparatus for metabolic determination so that it was possible to measure the exhaled amount of  $\text{CO}_2$  in a certain period.

As to the details of KROGH's apparatus the reader is referred to KROGH and LINDBERG (1931); here we shall merely briefly mention the principle of this volumetric method of oxygen determination. (Fig. 1 shows the modification of KROGH's apparatus used by the author.)

The animal is placed in a closed system consisting of an animal cage (Fig. 1 a) and a container with moist soda lime. (In Fig. 1 this has been replaced by the containers c, d, e, f and g (see later).) The cage and containers are immersed in a water bath the temperature of which is kept constant. By means of a pump the air is made to circulate. The velocity of the pump and thus of the air flow can be regulated by means of a rheostat. The carbon dioxide



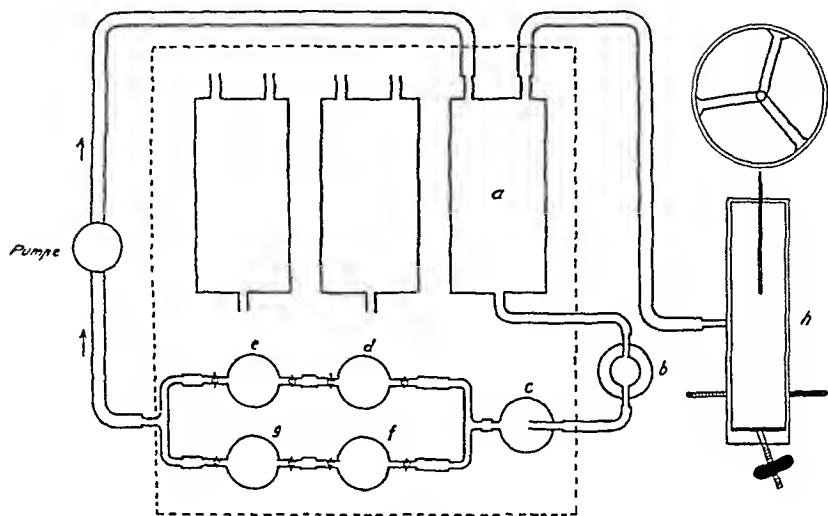


Fig. 1. The apparatus used by the author.

is absorbed by the soda lime and from a spirometer bell filled with oxygen and inserted in the system (Fig. 1, h) the oxygen requirement of the animal is covered. The oxygen consumption is registered on a kymograph.

## 1. Measurement of the Metabolism.

### a) Temperature of the Water Bath.

The temperature of the surroundings largely influences the size of the metabolism during metabolic measurement. Thus with low temperature of the surroundings rats have a comparatively high metabolism. With increasing temperature the metabolism decreases until the temperature is reached ("the critical temperature") at which the metabolism — other conditions being constant — again begins to rise. In ascertaining the standard metabolism it is necessary to take the measurements at the "critical temperature", and like the greater number of authors who have investigated these conditions I have made the experiments at 28°.

HEMMINGSEN (1933) has treated this subject in detail.

**b) Placing of the Rats in the Metabolic Apparatus  
Prior to the Measurements.**

All the experiments were made with rats who had fasted for 20—24 hours. In these 24 hours prior to the measurements the animals had been placed in the animal cage covered by a lid with wire netting. Consequently a rather large cage was selected ( $10 \times 10 \times 20$  cm). The cage has a loose bottom which can easily be taken out and cleaned and in order that the rats might not get wet a wire net was placed on the loose bottom. A bowl of water was put in the cage.

The reason why this procedure was chosen was that it was thought that the rats would then have had so long to accustom themselves to the new surroundings that they might be expected to keep quiet during the experiment.

**c) Body Temperature of the Rats.**

Rats are not completely homoiothermic, their body temperature depending in some degree on the surrounding temperature. DONALDSON (1924) and ISHIDA (1930) found that the body temperature of normal fasting rats may vary from  $34.8^{\circ}$  to  $38^{\circ}$  during metabolic experiments, even though the surrounding temperature was kept constant during the experiments.

HEMMINGSEN (1933) has closely described these conditions and concludes that in the case of rats it is necessary to correct the standard metabolism for body temperature, and for this correction he uses the values given by KROGH (1914) (5—6 % change of the standard metabolism for each degree of change in the body temperature).

Immediately after termination of the experiment the glass lid was removed, the rat taken up, and put under slight ether anesthesia.

A "rabbit thermometer" was then introduced into the rectum, care being taken that the thermometer reached right up to the liver. The reading was taken when the mercury column did not rise any more. The temperature measurements proved to lie rather close together. (Table 1.)

This is probably especially due to the fact that the animals in the rat stable were kept at a constant temperature, but it cannot probably be excluded with certainty that the circumstance that

the rats for about 24 hours before the experiments had been kept in the animal cage of the metabolic apparatus where the temperature was constantly 28° has contributed to stabilise the body temperature. As already mentioned, ISHIDA found that the body temperature of normal rats may vary more than 3 degrees. The cause of this great variation is no doubt the large temperature fluctuations of the rat stable in the material used by ISHIDA (from 6° to 29°). Thus ISHIDA's experimental conditions are different from those of the author and the results, therefore, cannot be compared.

Table 1.

*Body Temperature of Normal and Hypophysectomised Rats.*

	No. of investigations	Mean value	Highest value	Lowest value
Normal . . . . .	14	36.7	37.1	35.9
Hypophysectomised .	12	35.4	36.8	34.7

Further Table 1 shows that the body temperature of hypophysectomised rats lies about 1½° below that of the control animals. This is well known and has often been described.

#### d) Technical Remarks.

Rats display considerably less motility by day than in the night, and as it was of course endeavoured to keep the animals as quiet as possible during the experiments the glass lid of the animal cage was not covered. Other authors (MEYER and WERTZ, 1939) have even found it convenient to place an electric light above the cage.

In order to ensure that a period is chosen in calculating the metabolism in which the animal has remained perfectly quiet, a balloon device was at first used, which registered the movements of the animal. These were transferred to a Marey's tambour whose pointer thus traced a horizontal line on the drum in those periods in which the animal lay perfectly still. As one revolution of the drum takes about half an hour, the tambour had to be lowered (or raised) some millimeters every half hour. Since this arrangement was found inconvenient it was discarded, and instead a piece of the curve was selected in which the pointer of the spiro-

meter traced a straight line. Such a procedure must be regarded as quite safe. The spirometer pointer consists of a fine metal point which traces a very thin line on smoked paper. In periods in which the rat does not keep perfectly quiet an irregular line is traced.

Least currents of air (doors opened and shut or persons passing by) should affect the spirometer it is placed with the kymograph in a closed cellophane box with a glass cover.

#### e) Calculation of the Metabolism.

The drum is moved by a clockwork. The constant and even velocity of the drum was frequently controlled. The greatest variation found was less than 1 %. In view of the manner in which the calculation is made this constant velocity of the drum is of the greatest importance.

The oxygen consumption measured was reduced to 0° C and 760 mm Hg and was calculated by the following equation:

$$v = \frac{a \times b \times (B - W') \times 60 \times 10,000}{(1 + c') \times 760 \times S \times c \times d}$$

where  $v$  is the oxygen consumption per minute per sq. metre of surface.

$a$  is the number of mm which the spirometer has fallen in the selected piece of the curve, which is  $d$  mm long. In practice  $d$  was between 300 and 500 mm, corresponding to measurement of the metabolism within a period of 15 to 25 minutes.

$b$  is the number of cc. of oxygen represented by 1 mm change in the spirometer level (in the present case 4.45 cc.).

$B$  is the corrected barometric level.

$W'$  is the pressure of saturated vapours at  $t^\circ$  (room temperature).

$c$  is the number of seconds which the drum takes to move 1 mm.

$S$  is the surface of the rat measured in  $\text{cm}^2$ .

For the calculation of this quantity MEER's formula is used:

$S = K \times \sqrt[3]{W^2}$ , in which  $W$  is the weight of the animal in gm and  $K$  a constant different for the different animal species. The value of  $K$  for rats is given with some variation by different investigators. Thus RUBNER (1902) has given  $K = 9.1$ , a value later confirmed by BENEDICT (1938). According to CARMEN & MITCHELL (1926)  $K = 11.36$ .

A detailed account of these facts does not come within the scope of the present work; we need merely say that numerous other values have been used, and that those mentioned here seem to represent the extreme values.

In the present work the author has chosen RUBNER's constant (9.1) which was used as late as 1940 by HERRINGTON.

It should be added that small variations in this factor are of no great importance when an experimental material and a control material are compared.

## 2. Determination of the RQ.

### a) Investigations on the CO<sub>2</sub> Absorption.

In order to investigate the CO<sub>2</sub> absorption a constant amount of CO<sub>2</sub> per time unit was admitted to KROGH's apparatus. The procedure in these experiments was as follows: From a burette a sodium carbonate solution of known strength was allowed to drip into a flask with 4 n sulphuric acid. The CO<sub>2</sub> thus formed was admitted to the circulating air. If now the sodium carbonate solution was allowed to drip from the burette at such a rate that about 4 cc. of CO<sub>2</sub> was formed per minute (somewhat more than a large rat expires), and the container with soda lime was inserted and the animal cage left empty, a slight gradual rise of the spirometer was seen, a rise that ceased after some time, so that the kymograph now traced a horizontal line as a result of the fact that just as much CO<sub>2</sub> was now absorbed as was produced. This fact, that equilibrium is not brought about until there is a certain CO<sub>2</sub> tension in the apparatus gave rise to the following mathematical considerations.

Equilibrium sets in when the amount of CO<sub>2</sub> produced is equivalent to the amount absorbed. If we call the amount of CO<sub>2</sub> produced per minute  $p$ , the minute volume of the apparatus  $M$ , and the CO<sub>2</sub> percentage in the apparatus when equilibrium is present  $b$ , we get

$$p = \frac{b \times M}{100} \qquad b = \frac{p \times 100}{M}$$

that is to say the CO<sub>2</sub> percentage of the

$$\text{apparatus} = \frac{\text{the CO}_2 \text{ produced per minute} \times 100}{\text{the minute volume}}$$

From this it will appear that:

1) The  $\text{CO}_2$  percentage is independent of the volume of the apparatus,

2) It is important that the minute volume should be relatively large as the  $\text{CO}_2$  percentage in the apparatus will then be less.

In the determinations made by me the minute volume was c. 900 cc. and if we assume that a rat produces 4 cc. of  $\text{CO}_2$  per minute, the  $\text{CO}_2$  percentage in the animal cage will be less than  $1/2$ .

How long will it take before equilibrium is present, and on what does the time depend?

The amount of  $\text{CO}_2$  in the apparatus is called  $x$  and the variation in (addition to) the  $\text{CO}_2$  is called  $dx$ . The variation per unit of time is then  $\frac{dx}{dt}$ ,  $dt$  denoting a time interval.

$M$  is the minute volume;  $V$  the total volume of the apparatus;  $p$  is the amount of  $\text{CO}_2$  produced per unit of time. The amount of  $\text{CO}_2$  which leaves the apparatus and is absorbed per time unit is  $\frac{x}{V} \times M$ .

The variation in the  $\text{CO}_2$  content of the apparatus per time unit can also be expressed in terms of the difference between the  $\text{CO}_2$  produced per unit of time and the  $\text{CO}_2$  removed per unit of time. We then get the following equation:

$$\frac{dx}{dt} = p - \frac{x}{V} \times M$$

$$dt = \frac{dx}{p - \frac{x}{V} \times M}$$

This equation is solved with respect to  $t$

$$t = \frac{V}{M} \int_0^x \frac{1}{p \times \frac{V}{M} - x} \times dx = \frac{V}{M} \left( 1 \frac{p \times V}{M} - 1 \left( \frac{p \cdot V}{M} - x \right) \right)$$

or

$$t = \frac{V}{M} \times 1 \frac{1}{1 - \frac{M}{p \cdot V} \cdot x}$$

It will be seen that we are here concerned with an asymptotic function. In the case in question  $V$  was about 2000 cc.,  $M$  900 cc.

If  $p$  is assumed to be 4 cc. it will take 15.3 minutes before 99 % equilibrium is obtained.<sup>1</sup>

### b) The Apparatus for the Carbon Dioxide Absorption.

The expired air is first cooled in a condensor (Fig. 1, b) by which part of the water vapour is removed, and is then dried by being passed through the "desiccator", consisting of a  $5\frac{1}{2}$  cm wide tube with calcium chloride, at the mouth of which there is a 6 cm deep layer of Blaugel (Fig. 1, c).

Blaugel is a fine-grained silica gel to which an indicator has been added, the substance being a deep blue in the completely dry state. On absorbing water it turns first colourless, then pink. Blaugel is easily regenerated by being heated for a couple of hours in a drying box at  $160-170^{\circ}$ .

The dried air is now admitted to the absorption apparatus which consists of two large U tubes with a large expansion in the bend of the U tube. The tubes are closed with ground stop-cocks. (Fig. 1, d and c; Fig. 2).

By using U tubes of the form mentioned here two advantages are gained. 1) The volume of the tubes is considerably increased, 2) the flow of air through them is slower, on account of the expanded part.

The first U tube through which the air flows after desiccation contains rather coarse pieces of moist soda lime; the second one is filled with Blaugel and serves to absorb the moisture given off by the soda lime.

The two U tubes are weighed before and after an experiment. Since the tubes, especially the Blaugel tube, are charged with static electricity, it is necessary to wipe them with a moist piece of wash leather before weighing them. The increase in weight indicates the weight of the absorbed  $\text{CO}_2$ . Finally the air is passed over an indicator which is decoloured by  $\text{CO}_2$ .

That the Blaugel tubes are able to dry the air completely is proved by letting the apparatus function for 2—3 hours without animals and without any addition of  $\text{CO}_2$  but with a little water in the animal cage. The U-tube with Blaugel should then have increased just as much in weight as the soda tube has lost. The error in such weighing has never exceeded 3—4 mg. The increase

<sup>1</sup> For help in these calculations I am indebted to Mr ERIK JACOBSEN M. D., to whom I tender cordial thanks.

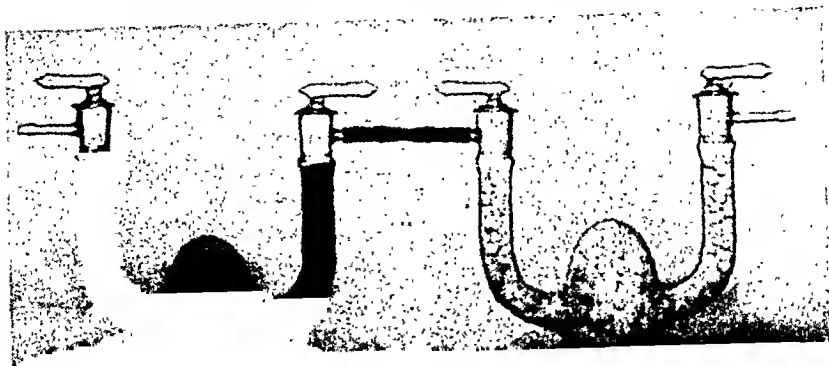


Fig. 2. The absorption apparatus (c.  $\frac{1}{4}$  of normal size).

— respectively loss — in weight of the two tubes in such experiments constitutes between 1 and 1.5 g, depending on the duration of the experiment and the length of time the soda lime has been used. It is necessary to use copious fresh Blaugel so as to get the air completely dried on both sides of the soda lime. For every second experiment the Blaugel was replaced by a new portion.

It should be noted that it was found that calcium chloride alone cannot be used at all for the quantitative drying instead of Blaugel, at any rate if such small amounts are used that the U tubes can be weighed on an analytic balance with an accuracy of  $\frac{1}{2}$  mg (maximum load 200 g), as done in the present investigation.

That the soda lime is able to absorb the  $\text{CO}_2$  completely will appear from the fact that experiments with considerable amounts of  $\text{CO}_2$  (about 20 cc. per minute) added to the circulating air never caused decolouration of the above-mentioned indicator.

With constant admission of  $\text{CO}_2$  to the apparatus a certain amount of  $\text{CO}_2$  will be accumulated — as previously stated — before the amount is supplied which is equivalent to the amount absorbed.

As it was desired to determine the oxygen intake and the carbon dioxide elimination within the same period as the RQ determination, the final  $\text{CO}_2$  absorption system must consist of two absorption systems, one of which is used to establish  $\text{CO}_2$  equilibrium in the apparatus (in the sequel called the equilibrium system) (Fig. 1, d and e). When equilibrium has been attained a change is made to the system in which it will be possible to recover



the amount of  $\text{CO}_2$  admitted in the period in which this system (the analytic system) has been used (Fig. 1, f and g).

The drying (Fig. 1 c) is common to the two systems. It has previously been mentioned that the  $\text{CO}_2$  percentage of the apparatus is inversely proportional to the minute volume, which again is dependent on the resistance in the circulation.

In order that the  $\text{CO}_2$  percentage of the apparatus may not change on changing from one system to the other it is therefore necessary that the pump used should be so constructed that the amount of air it conveys is, within reasonable limits, independent of the resistance in the apparatus. Further it is of course an absolute requirement that the pump should be perfectly tight.

#### c) Experiments Designed to Show the Possibility of Recovering a Calculated Amount of $\text{CO}_2$ .

The animal cage, desiccator, and both the absorption systems are lowered into a water bath where the temperature is kept constant. — The animal cage is empty.

The air is now allowed to circulate through the analytic system for a few minutes, then we change over to the equilibrium system in order as far as possible to empty the apparatus of  $\text{CO}_2$ , close the four stop cocks in the analytic system, take it out of the water bath, wipe it carefully at once, weigh it and put it in place again.

Then a known amount of  $\text{CO}_2$  per time unit is admitted to the apparatus. This is done as previously described by means of a sodium carbonate solution. The flask in which  $\text{CO}_2$  is formed was inserted in the apparatus in such a way that the sulphuric acid was constantly bubbled through since otherwise some  $\text{CO}_2$  would be dissolved in the fluid and of course more with an increasing amount of fluid. The sodium carbonate solution was standardised partly by titration with hydrochloric acid, and partly a certain amount of the sodium carbonate solution was admitted to the apparatus (without soda lime container) and it was ascertained that the amount of  $\text{CO}_2$  developed (increase on the kymograph) corresponded to the calculated amount of  $\text{CO}_2$ .

#### Experiment.

First the air current was passed through the equilibrium system and when equilibrium had been attained, a change was made — at a particular mark on the burette — to the analytic system.

For the next 45 minutes 10.0 cc. of sodium carbonate solution were now admitted, corresponding to 189.5 cc. of  $\text{CO}_2$ . Then the cocks in the analytic system were closed, and the analytic system was weighed. Table 2 shows the result of two such experiments.

Table 2.

Increase in weight of Blangcl tube	Loss in weight of soda lime tube	Absorbed amount of $\text{CO}_2$	Deviation from calculated amount of $\text{CO}_2$
467.6 mg	102.0 mg	365.6 mg or 186.5 cc	- 1.6 %
458.5 mg	80.3 mg	378.2 mg or 193.0 cc	+ 1.3 %

#### d) Discussion on the Standardisation of the Apparatus.

The usual way in which large apparatus for RQ determinations are standardised is by ethyl alcohol combustion. If the alcohol undergoes complete combustion the RQ for this substance is 0.67. For technical reasons it is, however, impossible to make such a determination with the apparatus here described, the maximal capacity of the spirometer being c. 500 cc. of oxygen, an amount which is able to burn c. 340 mg of alcohol. It was considered whether an attempt should be made to burn so small an amount of alcohol without a wick, *e. g.* by means of a red hot platinum wire, but since it was not certain that the alcohol would undergo complete combustion to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  by that method, the idea was given up.

#### e) The Procedure in the Animal Experiments.

We shall now briefly describe how the animal experiments were performed. The animal cage with the rat was inserted in the system. To ensure that the cage was saturated with water vapour at the temperature prevailing ( $28^\circ$ ) some of water, besides that in the drinking vessel, were placed in the bottom of the cage. This is a safeguard against the error in the oxygen determination that would arise if the animal voids the urine during an experiment, as it cannot be assumed that the moisture of the expired air is sufficient to saturate the circulating air with water vapour.

The air is now allowed to circulate through the analytic system for about 10 minutes, whereupon the cocks of this system are closed. It is taken out of the water bath, is dried, weighed, and put in place again. During this procedure the air circulates through the equilibrium system.

The spirometer is now filled entirely with oxygen and after the air has circulated through the equilibrium system for about 15 minutes, we change over to the analytic system at the same time as a mark is put on the kymograph (for instance by lightly touching the spirometer). When the oxygen bell is nearly empty, another mark is put at the same time as the cocks of the analytic system are closed. The experiment is now finished.

The whole spirometer bell has been used in all the experiments.

The analytic system is again weighed and the increase in weight is due to absorbed  $\text{CO}_2$ . The oxygen consumption is calculated from the distance between the two markings on the kymograph.

It is of the very greatest importance that the animal cage is frequently cleaned. At a certain stage of the investigations the RQ values obtained seemed unusually high, and blank tests showed that the soda lime tube gained much more in weight than the Baulgel tube lost, so that the  $\text{CO}_2$  values became too high. The cause of this was that  $\text{CO}_2$  developed in the cage from old urine. After the animal cage had been cleaned with a sodium hydroxide solution and water there were no discrepancies in weight between the two tubes in blank tests.

It was therefore necessary to discard the determinations that had preceded this discovery.

For each of the RQ values in this work a blank analysis was made after the determination; it proved satisfactory.

## Own Investigations.

### Method.

For the experiments were used albino rats descended from the stock of the Wistar Institute, and inbred in the Laboratory through 30—40 generations.

Only male rats were employed.

The rats were kept in the rat stables of the Laboratory — also for some time before the experiment — where the temperature is between  $20^\circ$  and  $22^\circ$ .

The diet of the animals consisted in

skimmed milk powder.....	22.5 p. c.
potato flour .....	40.0 p. c.
dried powdered pig liver .....	20.0 p. c.
dried yeast.....	7.5 p. c.
peanut oil .....	9.0 p. c.
shark liver oil .....	1.0 p. c.

Hypophysectomy was made by the parapharyngeal approach, as described by SMITH (1926, 1927).

The skulls of the hypophysectomised rats were saved for histological examination of serial sections through the decalcified brain base in the region of the pituitary fossa.

For hypophysectomy rats weighing about 200 gm were used.

## Results.

### 1) The Standard Metabolism.

From Fig. 3 it will appear that the standard metabolism of the control rats, measured at the collection of the oxygen, calculated per surface unit is independent of the weight of the animals, as has indeed been shown by BENEDICT (1938).

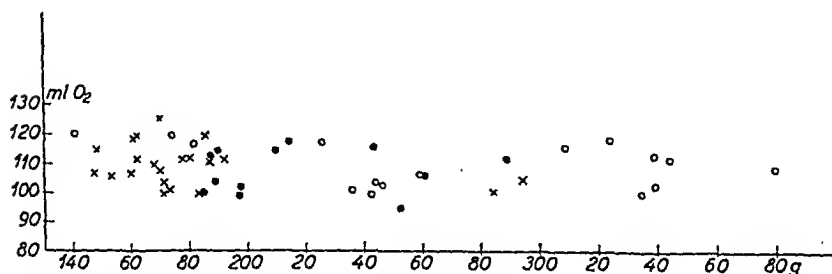


Fig. 3.

Abscissa: Weight of the rats (gm).

Ordinate: The standard metabolism (oxygen consumption) calculated per minute per sq. m of surface.

×. Untreated control rats.

●. Shamhypophysectomised rats.

○. Normal rats injected with Oleum arachidis.

Further, it is seen that there is no difference in the metabolism of untreated control rats, shamhypophysectomised rats, and normal rats treated with Oleum arachidis.

The values found here are in good accord with investigations by ANDERSON and COLLIP (1934), who found an oxygen consumption of 108—122 cc.

Fig. 4 shows the metabolic values for hypophysectomised animals.

It is seen that already 4 days after the operation there is a reduction in the metabolism of about 16 %. This reduction is not due to the operative trauma, since 4 shamhypophysectomised rats showed normal values on the third day after the operation (104—113—100 and 115 cc. of oxygen). After a week's hypophysectomy the reduction in metabolism is very marked, but with a considerable divergence be-

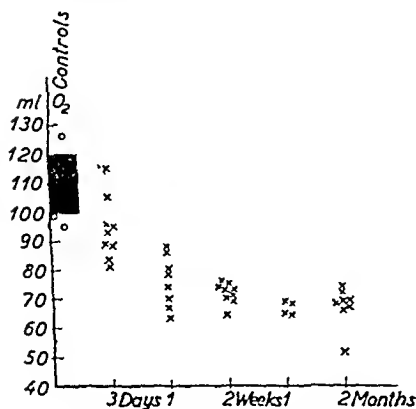


Fig. 4. Standard metabolism of hypophysectomised rats.

Abscissa: Time of hypophysectomy.  
Ordinate: Oxygen consumption (cc. O<sub>2</sub> per minute per sq. m of surface).

tween the individual determinations. Not until a week later are the values closer together, and the reduction in metabolism now amounts to 34 %. 1 or 2 months after hypophysectomy the reduction is 38 %.

These values accord with those stated by FORSTER and SMITH (1926) and TEAGUE (1939).

## 2) Determination of RQ.

The author's own experiments on the RQ determinations will appear from Fig. 5.

The determination was made after 24 hours' fasting. For the hypophysectomised animals the analysis was made between 2 weeks and 2 months after the operation.

The average value of the RQ for normal rats is 0.75, while for hypophysectomised animals the RQ is somewhat higher, 0.77.

A number of authors (FISHER and PENCHARZ, 1936; FISHER, RUSSELL and CORI 1936; RUSSELL 1938) found an increased

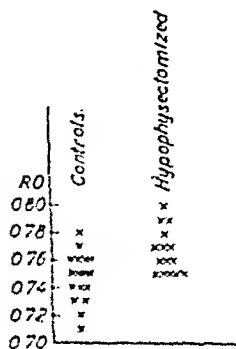


Fig. 5. RQ values.

respiratory quotient (0.78–0.81) in hypophysectomised rats, against 0.72 in normal animals. This finding has been made to subserve the theory that hypophysectomised animals have an increased carbohydrate combustion, as well as the theory that the abnormality of the fasting carbohydrate metabolism of hypophysectomised animals consists in the failure of these animals to form carbohydrate of fat.

Even though it has not been possible in this work to demonstrate the rather considerable rise in the RQ which has been mentioned above, it would seem, nevertheless, that the RQ of the fasting hypophysectomised rats has a tendency to lie somewhat higher than the RQ of the control animals.

### Summary.

A description of a simple apparatus for simultaneous measurement of standard metabolism and the respiratory quotient are given. The work is supplemented with investigations on normal and hypophysectomized rats.

The values obtained were in good accord with earlier investigations, even though it has not been possible to demonstrate the rather considerable rise in the RQ after hypophysectomy which has been described by other authors.

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## Glycerol Oxidation and Muscular Exercise.

By

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In 1944 E. HOLST demonstrated a very close correspondance between the metabolism of glycerol and that of ethyl alcohol. Like ethyl alcohol glycerol is fairly rapidly absorbed from the intestinal tract and evenly distributed. One to one and a half hour after oral administration even of large quantities of glycerol an equilibrium between blood and tissues is obtained. From this point the glycerol concentration in the blood declines following a rectilinear course, indicating that the oxidation of glycerol is independent of the concentration in the blood and tissues, at any rate within a fairly wide range. Since the urinary excretion plays a not quite insignificant part in the elimination of glycerol and since this excretion must be dependent on the concentration in the blood the elimination curve for glycerol cannot be absolutely rectilinear. However, for all practical purposes it can be considered so. The oxidation of glycerol as the oxidation of ethyl alcohol takes place exclusively or practically exclusively in the liver. In the peripheral tissues, especially the muscles, practically no direct oxidation occurs. The rate of disappearance in the liver is so high that only a fraction of the glycerol can be completely oxidized while the greater part must undergo only a partial oxidation. The product formed by this partial oxidation is assumed to be readily completely oxidized in the peripheral tissues.

As pointed out by LUNDSGAARD (1937) the predominant rôle of the liver in ethyl alcohol oxidation offers an explanation to



the fact that oxidation of ethyl alcohol is not affected by muscular exercise. During muscular work as in rest the primary partial oxidation in the liver is the limiting factor for the metabolism of alcohol, and the rate of this primary partial oxidation in the liver is assumed not to be influenced by the increase in oxidations in the working muscles.

Since, however, glycerol is a normal metabolite — in contrary to ethyl alcohol — it cannot be taken for granted that the partial oxidation of glycerol in the liver is not affected by muscular work. To maintain the conception that the liver plays a predominant rôle in fat metabolism it is necessary to assume that the fat metabolism of the liver as a whole is increased when muscular work is performed on a diet rich in fat. On the other hand to maintain the conception mentioned it is not necessary that the oxidation of glycerol should be increased during muscular exercise since the amount of glycerol oxidized per unit of time during rest after administration of glycerol far exceeds the amount of glycerol set free from fats even during strenuous muscular work performed mainly at the expense of fat.

For these reasons I have considered it worth while to try whether or how the elimination of glycerol is affected by muscular exercise.

### Experimental Part.

The experiments were carried out on two healthy young men in fairly good physical training, weighing 67 resp. 70 kilogrammes. The glycerol was administered per mouth in the morning 15 to 16 hours after the last meal. The quantity of glycerol administered has been 45 to 65 grammes dissolved in 300 ml. water. This quantity is somewhat larger than that used by HOLST in his experiments (0.54 gramme per kilogramme body weight). According to my findings, however, the fraction of the total mass of the body which acts as a solvent for glycerol to the same degree as does the blood is considerably larger than stated by HOLST. The mean value for this fraction, which after WIDMARK (1932) is denoted by  $r$ , HOLST has found to be 0.52 in human subjects. In one of my experimental subjects  $r$  was found to be about 0.70 and in the other even about 0.80. The reason for this discrepancy may be a systematic error in the determinations of the glycerol concentration in the blood, which is easiest explained as being due to a difference in the glycerol standards used by HOLST and me. The glycerol concentration in the standard stock solution is established by determination of the specific gravity of the solution. However, I have prepared my standard stock solution exactly in accordance with the directions of HOLST.

60 to 80 minutes after the administration of glycerol the first blood sample is collected. The following samples are drawn every 20 minute. The glycerol concentration is determined in 0.2 ml. whole blood by means of the method of HOLST (*loc. cit.*). 60 to 80 minutes after the first sample has been drawn the work is started. The work is performed on a KROGH bicycle ergometer. The intensity of the work has for both experimental subjects been 690 kgm/min. The duration of the working period has been 80 minutes. After the cessation of work the collecting of blood samples has been continued for 40 to 60 minutes. In the middle of the working period the gaseous exchange was determined using the DOUGLAS bag method.<sup>1</sup> From the results of these determinations the amount of fats metabolized during the period of work has been computed.

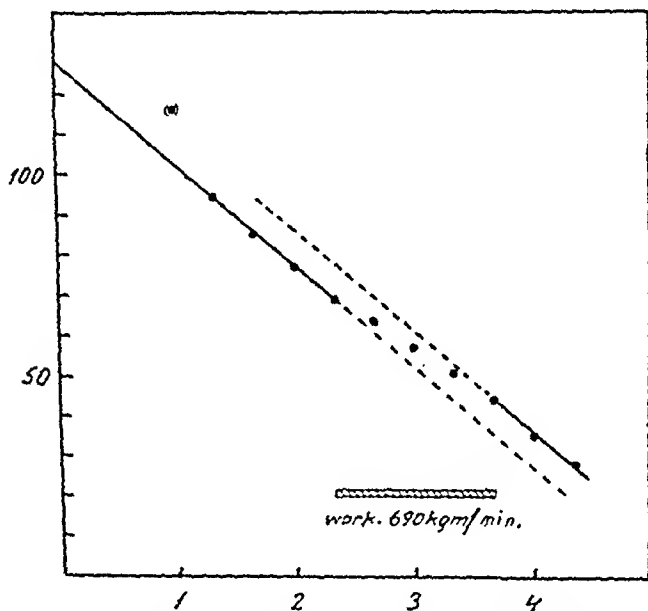


Fig. 1. Glycerol concentration in the blood after oral administration of 65 grammes of glycerol at zero time.

Abscissae: time in hours.

Ordinate: glycerol concentration in mg%.

Oxygen consumption during work: 1.515 liters/minute. RQ: 0.82 · 38.2 grammes of fat metabolized during period of work Displacement of curve corresponds to 4.5 grammes of glycerol.

The results from an experiment of this kind are given in fig. 1. As will be seen the decrease in blood glycerol concentration is definitely slowed down during the performance of muscular work. The displacement of the blood glycerol curve due to this effect of

<sup>1</sup> For the direction of this essential part of the experiments I am highly indebted to Dr. E. Asmussen.

muscular work stands out still more clearly when the experiments are carried out in that way, that the glycerol concentration in the blood is kept constant prior to the commencement of the period of work. This can rather easily be obtained since the amount of glycerol eliminated per unit of time by the experimental subjects can be calculated from the curves obtained after administration of a single large dose. In the experiment presented in fig. 2

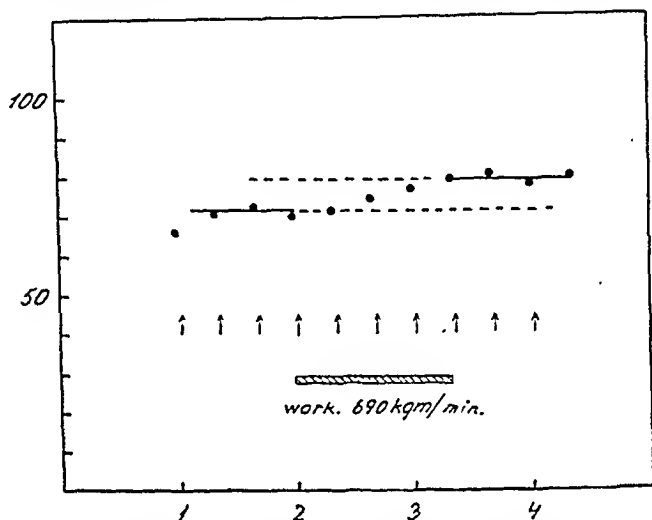


Fig. 2. Glycerol concentration in the blood after oral administration of 50 grammes of glycerol at zero time and 5.4 grammes at each of the small arrows.

Abscissae: time in hours.

Ordinate: glycerol concentration in mg%.

Oxygen consumption during work: 1.57 liters/minute. RQ : 0.85 · 33.8 grammes of fat metabolized during period of work. Displacement of curve corresponds to 4.2 grammes of glycerol.

50 grammes of glycerol were administered at zero time and 5.4 grammes were administered just after the drawing of each blood sample *i. e.* every 20 minute starting one hour after the first large dose.

The increase in the glycerol concentration in the blood in this experiment and the displacement of the elimination curve in the experiments of the other type may be due either to a decrease in the rate of glycerol oxidation in the liver or to the increased liberation of glycerol from fats during muscular work. A decrease in glycerol oxidation might be due to a competition between glycerol and other metabolites set free during muscular work. Glycerol set free in the metabolism must accumulate in the organism since the

enzyme system engaged in glycerol oxidation must be considered saturated after administration of glycerol.

If ethyl alcohol is administered after administration of glycerol the rate of glycerol oxidation is markedly decreased. An example of an experiment of this kind is presented in fig. 3.

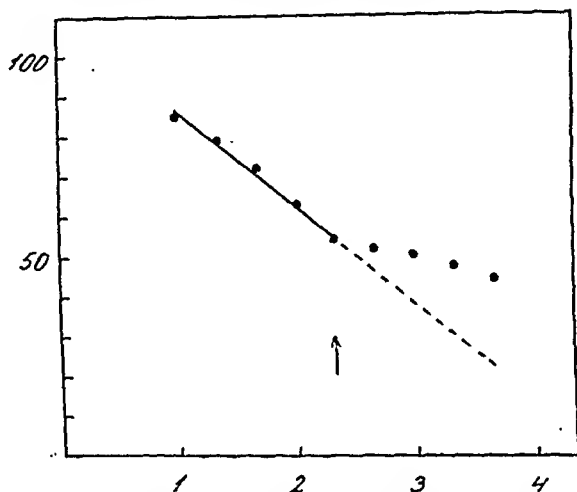


Fig. 3. Glycerol concentration in blood after oral administration of 65 grammes of glycerol at zero time. At arrow 50 grammes of ethyl alcohol per mouth.

Abscissae: time in hours.

Ordinate: glycerol concentration in mg%.

In this case the decrease in the rate of glycerol elimination is most likely due to a competition between the two substrates and thus further indicates the close relationship between glycerol and ethyl alcohol metabolism. It is not possible to suppress completely the oxidation of glycerol by administration of ethyl alcohol. In my experiments the rate of glycerol elimination was decreased to about  $\frac{1}{3}$  of the normal. However, it is not excluded that the oxidation of glycerol could be more completely blocked at very low glycerol concentrations. For this reason I have tried whether it is possible after administration of ethyl alcohol to demonstrate an increase in the normal very low glycerol concentration in the blood during muscular exercise. This, however, was not possible. I have only performed a single experiment of this kind. The blood glycerol concentrations found were: Before administration of ethyl alcohol: 2.2 mg%, after administration of ethyl alcohol but before start of the work: 2.0 mg%, during the period of work: 1.9 mg%, 3.6 mg%, 3.6 mg%, 3.0 mg% and 3.3 mg%.

### Discussion.

It is scarcely possible definitely to decide whether the effect of muscular exercise on the elimination curve of glycerol is due to: 1) an inhibition of glycerol oxidation, 2) the increased liberation of glycerol from fats during the period of work. However, I am inclined to consider the second explanation the most likely. A rather definite proof for the correctness of this explanation would have been obtained had it been possible to demonstrate an increase in the normal low glycerol concentration in the blood during muscular work after inhibition of glycerol oxidation by administration of ethyl alcohol. On the other hand the failure to demonstrate such an increase cannot be used as an argument against this explanation. From the determinations of the gaseous exchange the amount of fats metabolized during the period of work can be computed. Such calculations show that during 80 minutes of work about 3.5 grammes of glycerol have been liberated in my experiments. The normal rate of oxidation of glycerol after oral administration has in my experiments been found to be about 20 grammes per 80 minutes. Hence, even if the rate of glycerol oxidation is decreased to 17.5 % of the normal, the oxidation can keep pace with the liberation of glycerol during work and not until it is decreased to less than 10 % of the normal rate a definite increase in the concentration in the blood should be observable. When no increase in the normal glycerol concentration in the blood was observed it is presumably due to the fact that it has not been possible to suppress the oxidation of glycerol to a sufficient extent.

An argument in favour of the second explanation mentioned above can be found in the following fact. The quantity of glycerol necessary to bring about the displacements of the blood glycerol curves observed can be calculated when the fraction of the total mass of the body which acts as solvent for glycerol is known. This quantity has in my experiments been found to 3.5 to 4.3 grammes of glycerol. The accuracy of these calculations must be limited as the displacements of the curves observed are only small. However, the quantity of glycerol calculated in this way is in the same order of magnitude as the quantity of glycerol contained in the amount of fats metabolized during the period of work. If the slowing down of the fall in blood glycerol concentration should

be due to a depression of the glycerol oxidation it is unlikely that the quantity of glycerol which thus evades oxidation should correspond so closely to the quantity contained in the amount of fats metabolized.

If triglycerides are broken down in the periphery *i. e.* in the working muscles then at any rate the glycerol component has to pass to the liver to be partially oxidized before it can be utilized.

### Summary.

The fall in the glycerol concentration in the blood after oral administration of glycerol is slowed down during muscular work.

This effect is assumed to be due to the liberation of glycerol from the fats metabolized during the work.

If triglycerides are broken down in the working muscles, then at any rate the glycerol component is not directly oxidized in the muscles.

The presence of ethyl alcohol in the blood causes a marked depression in glycerol oxidation, a further indication of the close relationship between the metabolism of the two compounds.

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## On the Action of a Factor Interfering with the Chemical Determination of Thiamin by the Method of Melnick and Field. II.

By

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In a recent paper the author reported the occurrence in extracts of leaves and plants of a factor of phenol-like character which interfered with the chemical determination of thiamin by the method of MELNICK and FIELD (ÅGREN 1945 a). Some of the chemical properties of the factor were described. The present paper deals with the mode of action of the factor.

### Experimental.

*Preparation of extracts.* The factor was purified from extracts of aspen leaves according to the method previously described (ÅGREN 1945 a). About 0.5 mg of the purified and dissolved preparation caused the disappearance of 0.3 mg of thiamin when determined by the method of MELNICK and FIELD as applied by ÅGREN (1945 a).

*Thiamin determinations.* Thiamin was also determined by the thiochrome method, mainly according to the modification of HARRIS and WANG (1941). Special care was taken to work with the optimum amounts of potassium ferricyanide in the oxidation of thiochrome.

*Cysteine and cystine* were determined according to the method of KASSEL and BRAND (1938).

*Incubation procedure.* To 1 ml of a thiamin solution containing 2 micromoles of thiamin were added 2 ml of 0.03 M phosphate buffer of pH 7.4 and 1 ml of a solution containing the factor interfering with thiamin as determined with the diazo method. When necessary the

solution containing the factor was neutralized to pH 7.4. Water was then added to a final volume of 10 ml. 2 ml aliquots were analyzed according to the method of MELNICK and FIELD by adding 2 ml of ethyl alcohol, 1 drop of 0.5 N sodium hydroxide and 4 ml of the diazo reagent. 0.2 ml aliquots were usually analyzed by the thiochrome method. The time of incubation, *i. e.* the time taken to make up the 10 ml solutions, to remove the different aliquots and to make the final addition of the diazo and thiochrome reagents amounted to about 4—5 minutes. The procedure was carried out at 20° C.

## Results.

The activity of a 5 per cent solution of the factor purified from aspen leaves was determined by the method of MELNICK and FIELD.

A. In a dilution 1 : 10 the extinction value was 0.

B. In a dilution 1 : 50 the extinction value was 0.16.

C. A control without the factor gave the extinction value 0.45. The possibility existed that the interference of the factor might be associated with a greater affinity of the diazo reagent for the factor than for the quaternary nitrogen in the thiazole nucleus of thiamin (cf. MELNICK and FIELD 1939). Determinations were therefore carried out with 0.5 ml aliquots of solution B + 1.5 ml of water + 2 ml alcohol and 4 ml of the diazo reagent. The extinction value 0.03 was obtained. Calculated value  $\left(\frac{B}{4}\right) = 0.04$ . As a control 0.5 ml aliquots of C were diazotized in the same manner. The extinction value 0.11 was obtained. Calculated value  $\left(\frac{C}{4}\right) = 0.11$ . Obviously the factor interfered with the thiamin determinations even in the presence of a considerable excess of the diazo reagent. The result did not provide evidence of a competition for the reagent between thiamin and the factor.

On the other hand it was also possible that the activity of the factor might be associated with a direct change of the thiamin molecule. This alternative was perhaps favoured by the reducing properties of the factor, which might change the thiamin molecule in a way similar to that of sodium sulphite (WILLIAMS et al. 1935) or cysteine and glutathione (ÅGREN 1945 b). The possibilities were first investigated by means of the *Phycomyces* assay method. The result of this investigation, to be published in detail elsewhere, showed that the growth of the fungus was also normal



on a medium where the thiamin content, according to determinations by the MELNICK and FIELD method had been totally destroyed by the addition of a sufficient amount of the leaf factor.

Considering the result previously obtained with the MELNICK and FIELD method, the outcome of the *Phyeomyces* assay might be explained in two ways. With regard to the reducing properties of the factor the most probable alternative seemed to be that the factor acted in a manner similar to that of the thiamin-destroying fish enzyme or sodium sulphite, in which case the fungus, after coupling together the two halves of the vitamin, would grow as normal. A second possible explanation, also in line with the reducing property of the factor, would involve a change of the thiamin molecule into a reduced state where it would not couple with the diazo reagent. In view of the results obtained with the *Phyeomyces* assay method this alternative seemed less probable since LIPMANN and PERLMANN (1938) found that their reduced form of the vitamin was biologically inactive. However, the biological method used by these authors was not described.

To determine whether thiamin was split or only reduced by the action of the leaf factor, the reaction was followed by means of thiochrome determinations (HARRIS and WANG 1941). In the modification used by these authors no fluorescence was obtained in tests with 100  $\mu$ g of either or both of the two halves of the vitamin. Thiamin was also split with sodium sulphite according to MASON and WILLIAMS (1942) in the following way:

I. 2 micromoles of thiamin + 25 mg of sodium sulphite dissolved in 3 ml of water. pH adjusted to 5.0. 2 ml of 0.2 M acetate buffer of pH 5.0 added.

II. As in I but with 0.2 micromoles of thiamin.

The samples were incubated for 15 minutes at 100° C and subsequently diluted to 10 ml. 1 ml aliquots gave no trace of blue fluorescence in the thiochrome method. The MELNICK and FIELD reaction carried out on 2 ml aliquots of I and II was negative. Determinations with the thiochrome method were next carried out on 0.5 ml and 0.1 ml samples of solutions made up as in A and C (p. 35). In both series the same fluorescence was obtained when the aliquots from A and C were compared. The

\* The writer wishes to express his thanks to Professor MELIN of the Department of Physiological Botany for 2,5-dimethyl-4-aminopyrimidine and 4-methyl-5- $\beta$ -hydroxyethylthiazole (Merck) used in the present investigation.

factor obviously did not act by splitting thiamin into its two halves.

When 15 mg of cysteine or vitamin C was incubated at pH 7.4 with 2 micromoles of thiamin for 2 hours at 40° C (total volume 10 ml) the MELNICK and FIELD reaction carried out on 2 ml aliquots was negative (cf. ÅGREN 1945b). Thiochrome determinations simultaneously carried out on 0.2 ml aliquots gave the same fluorescence as aliquots from a control series made up in the same way but without cysteine or vitamin C. Control series containing only vitamin C gave no blue fluorescence, but those containing cysteine only, gave a distinct blue fluorescence as shown in Table 1.

Table 1.

*The influence of cysteine and vitamin C on determinations of thiamin carried out with the Melnick-Field method and the thiochrome method.*

2 micromoles of thiamin incubated for 2 hours at 40° C. with the reducing substance. Total volume in each series 10 ml. The diazo reaction was carried out on 2 ml aliquots and the thiochrome reaction on 0.2 ml aliquots.

S a m p l e	Thiamin in per cent of original found by	
	diazo method	thiochrome method
Cysteine 15 mg + B <sub>1</sub> . . .	0	105
Cysteine 15 mg (control) . .	no colour	blue fluorescence corresponding to about 5 % of thiamin tests
Vitamin C 15 mg + B <sub>1</sub> . . .	0	100
Vitamin C 15 mg (control) . .	no colour	no fluorescence
B <sub>1</sub> (control) . . . . .	100	100

In the series with cysteine + thiamin, cystine precipitated during the reaction. No cysteine was recovered in the control series.

It seemed conceivable that a possible reduced form of thiamin might be oxidized to thiocrome with ferricyanide and alkali. In the form obtained after interaction with cysteine or vitamin C the thiamin could not be determined by the method of MELNICK and FIELD. As reported by LIPMANN and PERLMANN, sodium hyposulphite reduced thiamin. When the reaction was performed in a manner similar to that described by the authors a product could be obtained which reacted with the diazo reagent of MELNICK and FIELD (ÅGREN 1945b). The process was repeated exactly as previously described and was followed by determinations by the thiochrome and diazo methods. The thiochrome determinations were carried out on 0.2 ml aliquots of the solutions and the diazo

Table 2.

*The interaction between thiamin and hyposulphite.*

2 micromoles of thiamin incubated for 2 hours at 30° C with different amounts of hyposulphite. The thiamin values are given in per cent of the original amounts present. TM = thiochrome method. DM = diazo method.

S a m p l e	Thiamin values after incubation with the following amounts of hyposulphite in millimoles $\times 10^{-2}$							
	0		1		4		8	
	TM	DM	TM	DM	TM	DM	TM	DM
B <sub>1</sub> + Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub> in NaHCO <sub>3</sub> . . . . .			100	100	50	55	15	10
B <sub>1</sub> in NaHCO <sub>3</sub> . . . . .	100	98						

determinations on 2 ml aliquots diluted to 10 ml. Typical values are given in Table 2.

When the molar ratio between thiamin and hyposulphite was 1 : 5 no inactivation of thiamin as determined by the two methods could be found. When greater amounts of hyposulphite were used the "inactivation" observed was probably associated with a further reaction of another type, also observed by LIPMANN and ascribed by him to the saturation of a second double bond in the thiamin molecule. Furthermore, it was not possible to reoxidize the last-mentioned type of reduced product with ferrieyanide and alkali (cf. Table 2).

From the results reported above it seemed possible that a reduction of thiamin could occur in several steps or in different ways, an observation already made by LIPMANN in 1937. The latter author observed a transient yellowish-green colour appearing on the addition of hyposulphite to neutral thiamin solutions. According to LIPMANN the hydrogenation of the first double bond in the thiamin molecule proceeded in two steps and a further reaction was connected with the saturation of a second double bond. The last mentioned reaction was stated to be an irreversible step. From the results obtained in the present investigation it seemed probable that the reduction obtained with hyposulphite was of a type other than that produced by vitamin C and cysteine (cf. SEALOCK and GOODLAND 1944) and probably by the factor isolated from aspen leaf. In the first case the reduced vitamin could be determined by the MELNICK and FIELD reac-

Table 3.

*The interference of different benzene derivatives containing phenol or quinone groups on the determination of thiamin by the Melnick-Field method.*

2 micromoles of thiamin and 15, 1.5 or 0.50 mg of the substance to be tested were dissolved in a volume of 10 ml. The values refer to the extinction values given by diazotized 2 ml aliquots of the 10 ml samples.

Substance	Formula	Extinction values in the presence of		
		15 m	1.5 mg	0.50 mg
Phenol <sup>1</sup>	$C_6H_5OH$	0.46	0.45	0.45
Thymol <sup>1</sup>	$CH_3(C_6H_4)C_6H_4OH$	0	0.40	0.45
Pyrocatechol <sup>1</sup>	$C_6H_4(OH)_2$	0	0	0.50
Veratrole <sup>1</sup>	$C_6H_4(OCH_3)_2$	0.46	0.45	0.45
Resorcinol <sup>1</sup>	$C_6H_4(OH)_2$	0	0	0.45
Orcinol <sup>1</sup>	$CH_3C_6H_4(OH)_2$	0	0.46	0.45
Hydroquinone <sup>1</sup>	$C_6H_4(OH)_2$	—	0	0.55
Hydroquinone-ethyl-ether <sup>2</sup>	$C_6H_4(OCH_2CH_3)_2$	0.45	0.46	0.45
Quinone <sup>1</sup>	$O=C_6H_4=O$	0	0.50	0.40
Pyrogallol <sup>1</sup>	$C_6H_3(OH)_3$	0	0	0.50
Phloroglucinol <sup>1</sup>	$C_6H_3(OH)_3$	0	0.18	0.55
$\alpha$ -Naphthol <sup>1</sup>	$C_{10}H_7OH$	0	0	0.46
Naphthoresorcinol <sup>2</sup>	$C_{10}H_7(OH)_2$	0	0	0.45
$\alpha$ -Naphthoquinone <sup>2</sup>	$C_{10}H_6O_2$	0.10	0.50	0.45
Vanillin <sup>1</sup>	$CH_3O(OH)C_6H_4CHO$	0.45	0.45	0.44
Haematoxylin <sup>2</sup>	$C_{18}H_{14}O_6$	0	0	0.55
Quercetin <sup>1</sup>	$C_{15}H_{10}O_7$	—	0.50	0.45
Borneol <sup>1</sup>	$C_{10}H_{18}OH$	0.40	0.45	0.46

<sup>1</sup> Kahlbaum preparation.

<sup>2</sup> Merck preparation.

tion, but not after interaction with factors of the second type. In a comparison of the effects of several phenols and closely allied compounds it therefore seemed preferable to use diazo method.

The series was set up in the following way. 45 mg of each substance was dissolved, when necessary with the aid of small amounts of ethyl alcohol to a final volume of 3 ml. Dilutions of 1:10 and 1:50 were made. 1 ml of the thiamin solution containing 2 micromoles vitamin B<sub>1</sub> + 2 ml. 0.05 M phosphate buffer of pH 7.4 was mixed with 1 ml of one of the solutions containing the concentrated or diluted phenol. The volume was brought to 10 ml by addition of distilled water. Without further incubation 2 ml samples were diazotized. Usually about 4—5 minutes elapsed before the addition of the diazo reagent. The results are given in Table 3.

## Discussion.

In the analysis of the mode of action of the factor present in leaves and interfering with the determination of thiamin according to the method of MELNICK and FIELD, two possibilities were first excluded. There was no competition between the factor and the vitamin for the reagent, nor did the former split the thiamin molecule into its two halves, pyrimidine and thiazole. The most probable explanation seemed to be that the factor, by virtue of its reducing property converted the vitamin into a reduced state.

The reduction of the vitamin has previously been studied by LIPMANN and PERLMANN. These authors, after treating the substance with sodium hyposulphite or with hydrogen and platinum black, considered that they obtained a reduced form of thiamin. However, they did not succeed in reoxidizing the vitamin to thiochrome with ferrieyanide and alkali. They also stated that the biological activity was lost in their hydrogenated product.

By estimation with the diazo and the thiochrome methods it has now been demonstrated that the reduction occurring on treatment with sodium hyposulphite is probably of a type other than that produced by the action of cysteine, vitamin C and the leaf factor. In the first case a product was obtained which could be determined quantitatively by the diazo method. In the three other cases products were obtained which could be determined only with the thiochrome method. Another important difference seemed to be that the product obtained after complete interaction of the leaf factor and thiamin was biologically active, in contrast to the result obtained by LIPMANN and PERLMANN with hyposulphite.

LIPMANN and PERLMANN made several attempts to isolate their hydrogenated thiamin product. They met with great difficulties and did not succeed, probably owing to the inhomogeneity of their reduced material. No attempts have so far been made to isolate a possible reaction product of thiamin and the leaf factor.

The investigation of a series of phenols and allied compounds demonstrated that several of them, especially when they contained two or more phenol hydroxyl groups and had reducing properties, reacted at a similar concentration and in a manner similar to the leaf factor. Compounds containing only methoxyl groups or

quinones were inactive or only slightly active. These results also support the theory that the active factor in isolated material from aspen leaf is a phenol-like substance which, by reducing thiamin, interferes with the MELNICK and FIELD method for the determination of the vitamin. In this connection it is interesting to mention the results of TOMARELLI and GYÖRGI (1945), who found that phenol compounds such as vitamin E inhibited by their reducing properties the oxidation of carotene to a biologically inactive state. It may be that a hypothetical function of the leaf factor is associated with the reducing properties of the phenol-like compound. Finally, the results reported in this paper demonstrate the obvious advantage of the thiochrome method over the diazo method for the determination of thiamin when reducing substances are present.

### Summary.

The highly purified material from aspen leaves contains a substance or substances with reducing properties and phenol-like character which seem to interfere with the chemical determination of thiamin by the reaction of MELNICK and FIELD. The vitamin is apparently reduced to a state which is biologically active. Thiamin which has interacted with cysteine, vitamin C or the phenol-like leaf factor can be determined with the thiochrome method. Cysteine gives a faint blue fluorescence in the thiochrome test.

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## The Fluorescence Microspectrographic Localization of Riboflavin (Vitamin B<sub>2</sub>) and Thiamin (Vitamin B<sub>1</sub>) in Tissue Cells.

By

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In a previous publication (SJÖSTRAND, 1944) the autofluorescence of a number of organs was subjected to examination by means of an improved method of fluorescence microscopy. Nowadays, sources of light with great ultra-violet intensity per surface unit are at our disposal and, accordingly, suitable for fluorescence microscopy. For this reason, attention was directed entirely to the elaboration of a more appropriate method of dealing with specimens in order to obtain, *inter alia*, a more distinct image by increased resolving power than hitherto achieved at fluorescence microscopy. This is a prerequisite for utilizing the fluorescence in order to obtain a careful cytologic localization of biologically significant substances with a characteristic fluorescence.

The localization of the fluorescent substances normally present in the tissues was considered to be of primary interest. The principal aim was, therefore, to try to maintain as far as possible during preparation the true localization of these substances in the cells.

The ALTMANN freezing-drying method for fixation was found to be the most suitable fixation technique. The possibility of reducing the thickness of the sections down to 2—3  $\mu$  gave an increase in the resolving power, facilitating a careful cytologic localization of fluorescent substances. The risks of a diffusion of the fluorescent substances (with the exception of fat and lipoids)

and a change in their localization are diminished owing to the fact that the specimens do not have to become exposed to the influence of another medium than paraffin — which also could be avoided.

The forementioned publication was mainly of a descriptive kind. In view of the lack of a more exact method of analysis, the chemical nature of the fluorescent substances has not been subjected to discussion.

Continued investigations have been concentrated on the elaboration of a fluorescence microspectrographic method of analysis for identifying, in the first place, riboflavin and thiamin.

In 1913, LEHMANN drew attention to the possibilities of utilizing a fluorescence microspectroscopic analysis. In 1925, POLICARD made a few attempts to identify haematoporphyrin in tissue sections by means of such a method.

BORST and KÖNIGSDÖRFFER (1929) described an apparatus for fluorescence microspectroscopy, by means of which they localized porphyrin in tissue sections from a case of porphyria congenita. The same apparatus was used by FIKENTSCHER (1931) for the localization of porphyrin in the skeleton at ochronosis and in porphyria in animals.

The characteristic fluorescence spectra of the porphyrins render them particularly suitable for such an analysis which was, moreover, facilitated by the large quantities of porphyrin occurring in the tissues under these pathological conditions.

Other authors, such as BOMMER (1929), have stated that (see page 427) continuous spectra without characteristic maxima are obtained at the fluorescence microspectroscopy of tissue sections and have therefore failed to utilize this method.

JANSEN (1942) worked out a visual microspectrophotometric method, using large slit widths on account of the insignificant light intensity.

V. EULER, HELLSTRÖM and ADLER (1935) attempted to localize riboflavin by means of fluorescence microspectrography in the eyes of fish, basing their experiments on V. EULER'S and ADLER'S discovery of great quantities of riboflavin in this organ. They employed a direct-vision hand spectroscope with a camera and directly compared spectrograms from the vitreous body, containing diffused riboflavin and from riboflavin solutions. In both cases, a fluorescence maximum was obtained at about 550 m $\mu$ . However, the above-mentioned authors did not perform a more



detailed analysis by measuring the spectrograms. The spectrography was facilitated in this particular case by the lack of auto-fluorescence in the vitreous body.

### Method.

The fluorescence spectra of the organic substances used in this connection are band spectra, frequently with relatively broad bands. Under these circumstances, a more detailed analysis of the fluorescence spectrum, with a careful determination of the relative distribution of the intensity in the spectrums, and thereby, also of the position of the maxima, is a prerequisite with regard to a more exact identification of the fluorescent substances.

On account of the insignificant intensity, the fluorescence spectrum has been registered photographically, in the course of which miniature spectrograms have been recorded by means of a miniature camera (Leica) on a 35 mm film (Agfa Isopan Ultra).

In addition, spectra from an incandescent lamp (Osram, type Wi 34, 40 Watt), serving as a standard of comparison, have invariably been photographed on the same film in a series of different intensities, and, with a time of exposure identical with that employed in the taking of the fluorescence-spectrograms, in order to avoid having to consider the Schwarzschild-exponent. The intensity was varied by the use of a series of density filters with known extinction or by logarithmic step sector.

Density curves have in this way been possible to construct at all the wave lengths where measurements had been performed, and the distribution of relative energy of the fluorescence spectrum, in relation to the incandescence lamp, could thus be determined.

Since the color temperature of the standardized incandescence lamp operated at this voltage is  $2900^{\circ}\text{K}$ , its relative spectral distribution of energy has been calculable with the aid of the Wien radiation law. The energy distribution in the fluorescence spectrum has then been recalculated in relation to a spectrum with an even energy distribution.

The different spectrograms have been subjected to photometry by means of a recording microphotometer (constructed according to M. SIEGBAHN).

An ordinary microscope has been used with a spectroscope connected with the ocular, constructed so as to enable the spectrography of isolated structural details in the field of vision.

Philip's super-high-pressure mercury-burner The Philora S P 500 has been employed as a source of light. Only the violet and long-wave ultra-violet-parts in the mercury spectrums have been used, the other parts having been filtrated away by means of a series of UG filters (Schott & Gen., Jena) and a copper-sulphate filter. In order to avoid fluorescence in the lenses, a microscope condenser of quartz was applied, as well as euphros glass filters, inter alia, in front of the anterior lens in the objective.

On account of the fact that a great number of biologically valuable substances are rapidly destroyed by the radiation, the apparatus has been constructed so as to permit the addition of a number of exposures from identical structural details in one and the same spectrogram. The total time of exposure has varied between 5 and 80 seconds, which shows the considerable light economy of the apparatus.

The specimens have been submitted to freezing-drying fixation and embedded in paraffin.

Also an apparatus for fluorescence microscopy and microspectrography at low temperatures about  $-180^{\circ}\text{C}$  has been built.



Fig. 1. Frog's eye, a fluorescence microphotograph. From the left to the right, ganglion cell layers, visual cells, pigment epithelium and choroid membrane. Magnification 350  $\times$ .

### Riboflavin.

The observations made by v. EULER and collaborators, regarding the occurrence of large quantities of free riboflavin in the pigment epithelium of certain fish and mammalian eyes, have formed the basis for a test of the usefulness of the fluorescence-microspectrographic method for the localization of riboflavin, from a histological as well as a cytological point of view.

In the first place, frog's eyes (*Rana temporaria*) have been used, cut into sections of a thickness of 3–5  $\mu$ . It is possible on these sections, to perform a careful and detailed fluorescence-microscopic examination by means of the forementioned method without any staining or previous treatment whatsoever (Fig. 1).

When micro-drops of normal acetic acid are placed on the choroidea an intensive, yellow-green fluorescence is obtained from the drops. This reaction does not set in in the pigment epithelium, the bacillary layer, or the ganglion cells.

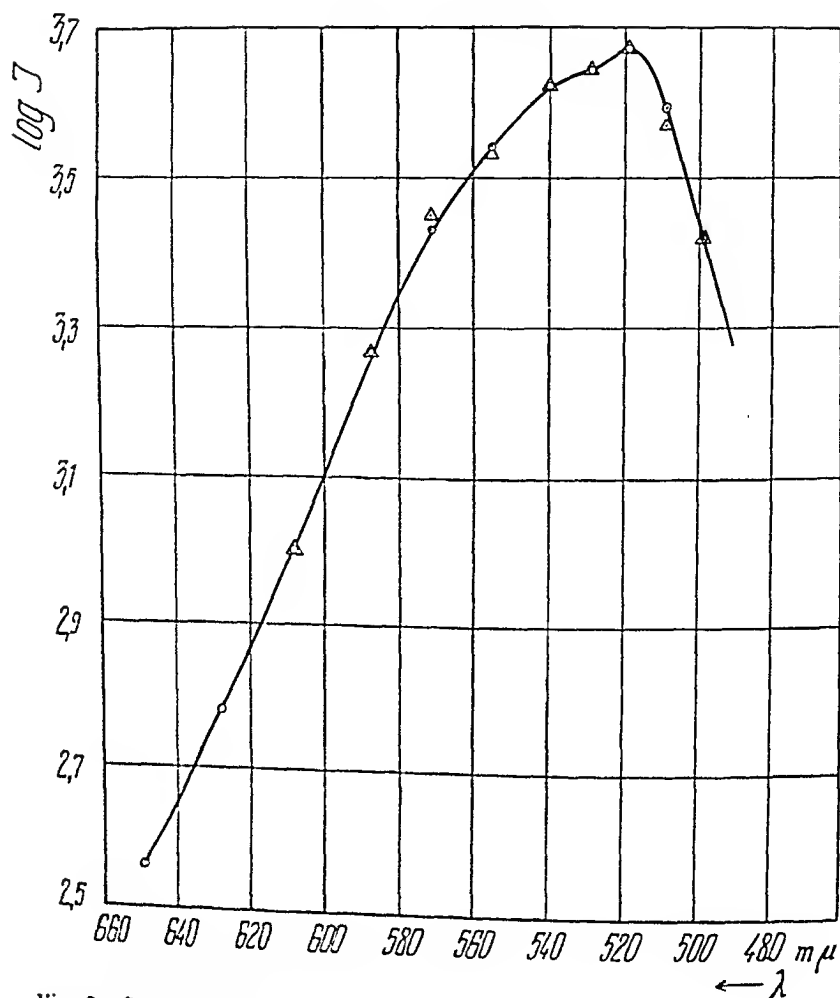


Fig. 2. Quantitative fluorescence spectrum of a riboflavin solution in normal acetic acid, denoted by circles, and of riboflavin in the vascular membrane in a frog's eye at microextraction with normal acetic acid, denoted by triangles.

Fig. 2 shows the fluorescence spectrum of a riboflavin solution in normal acetic acid and of the yellow-green fluorescent substance in the choroid membrane at the forementioned drop reaction. The conformity is so exact as to justify the conclusion that the yellow-green fluorescent substance in the choroid membrane is riboflavin.

This conformity prevails also with regard to the  $pH$ -dependence of the fluorescence, and the reversible reducibility with reversible loss of the fluorescence, etc.

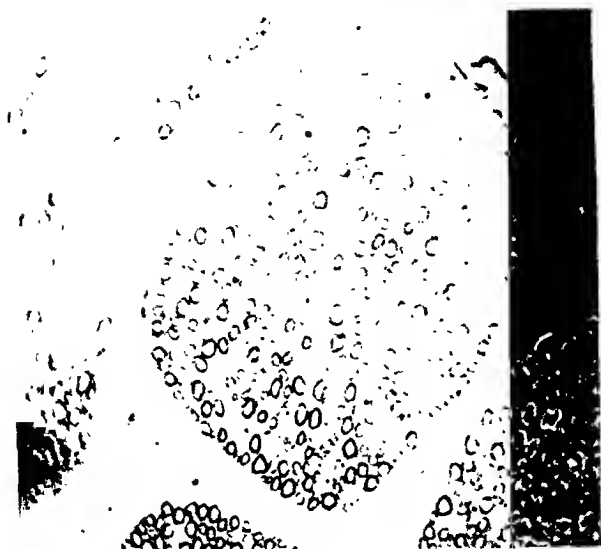


Fig. 3. The sciatic nerve of a rabbit, a fluorescence microphotograph. Strong bluish-white fluorescence from the myelin sheaths. Magnification 500  $\times$ .

The investigations with macrochemical methods, which have revealed conspicuously great quantities of riboflavin in the eyes of different animal species, further confirm this conclusion.

The position of the maximum of the fluorescence band at 520  $m\mu$  is not in agreement with earlier data which, however, also vary. As a rule, it is stated to lie at 560—565  $m\mu$  (KARRER et al., 1934, BERRY and GOUZON, 1935) but EYMERS and VAN SCHOUWENBURG, 1937 have localized the maximum to 545  $m\mu$ . Since the earlier determinations have not been made at the same  $p_H$  as in this particular case, the differences are probably due to a deviation of the maximum in relation to the  $p_H$ . Such a deviation of the maximum has been ascertained with regard to lumichrome which, in acid solution, has a maximum of 462  $m\mu$  and, in alkaline solution, of 547  $m\mu$  (KARRER et al.). An admixture of lumichrome, which might be formed during the exposure, would also serve to explain the deviation of the maximum towards a shorter wave-length.

The quantity of riboflavin may vary from one frog to the other. When abundant, fluorescence microscopy may reveal yellow-orange, fluorescent granules of riboflavin in the choroid membrane. At about  $-180^\circ C$  these granules assume a red fluorescence. This is in agreement with DHÉRÉ and CASTELLI's (1938) observa-

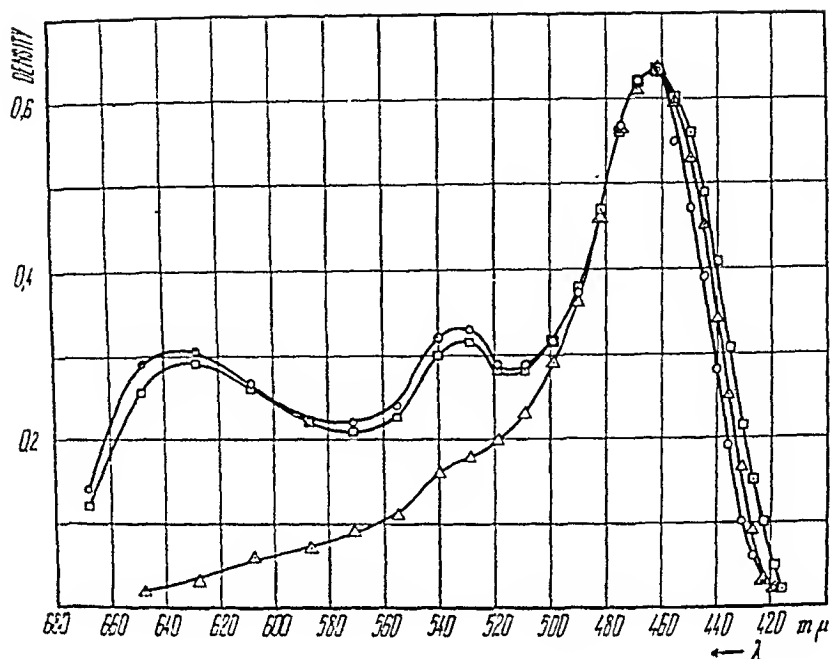


Fig. 4. Photometric curves of the fluorescence microspectrogram of a thiamin solution, denoted by circles, of the myelin sheaths in the sciatic nerve of a rabbit, denoted by squares, and of thiochrome, denoted by triangles. The spectrograms are mutually comparable, having been registered under identical conditions on the same film. See further the text.

tions on riboflavin crystals. Examinations of the fluorescent spectrum of these granules at  $-180^{\circ}\text{C}$  are, at present, being carried on at the institute.

Thus, the localization of riboflavin in this case is not in the pigment epithelium but in the choroid membrane.

Furthermore, according to preliminary experiments, linked riboflavin seems to be localized, *inter alia*, to the basal rod structures in the proximal convoluted tubule of the renal tubuli, i. e. localized to a definite cellular structure.

### Thiamin.

In the earlier work in fluorescence microscopy, the author described an intensive, bluish-white fluorescence localized to the myelin sheaths (Fig. 3).

At fluorescence microspectrography of the sciatic nerve of rabbit similar spectrograms were obtained from the myelin sheaths

and from a thiamin solution. Photometric curves of the spectrograms are reproduced in Fig. 4. In the same diagram, also the corresponding curve of thiochrome is described. These photometric curves do not, as a matter of course, offer any definite information regarding the localization of the fluorescence maxima. However, a comparison between them is justified owing to the fact that all the spectrograms have been registered on the same film under identical circumstances.

A band with a maximum at about 660  $m\mu$  can be noted in all the spectrograms. This is in good conformity with KUHN's and VETTER's (1935) determination of the localization of the fluorescence band with regard to thiochrome. This common maximum has been attributed to the thiochrome. In addition, a certain fluorescence intensity at lower wave-lengths is to be found above all in spectra from myelin sheaths and thiamin solutions. Since thiamin is stated to lack fluorescence, this capacity can no doubt be attributed to other fluorescent components formed by the thiamin. Such components have been determined by KINNERSLEY, O'BRIEN and PETERS (1935), among others.

The bluish-white fluorescence from the myelin sheaths is relatively weak immediately after the fixation, but increases when the sections have been left for a certain time and, particularly, when kept in heat (60—70° C). This indicates a spontaneous oxidation of thiamin to thiochrome, which is also known to take place with regard to pure thiamin preparations.

Thus, thiamin can, for the greater part, be localized to the myelin sheaths in the peripheral nerves.

v. MURALT (1934) attempted to localize thiamin in the peripheral nerve fibres by the thiochrome reaction. At the oxidation of frozen sections with 1 per cent of kaliumferricyanide in an alkalic solution (n/10 Na OH), an intensive blue fluorescence was obtained, which was assumed to derive from thiochrome, judging from a series of indirect indications supporting this theory. Thiamin should, according to these examinations, be localized to myelin sheaths. However, the method adopted did not exclude the possibility of a changed position owing to diffusion. A similar objection cannot be raised with regard to the analysis dealt with here.

At an examination of the co-carboxylas-effect in the myelin sheath and in the axis-cylinder substance, respectively isolated from squid nerves, NACHMANSOHN and STEINBACH (1942) noted

many times greater activity in the myelin sheaths than in the axis-cylinders.

### Discussion.

There is justification to assume that also fluorescent substances other than porphyrins, which are conspicuous thanks to their particularly characteristic fluorescence spectra, are ascertainable and possible to localize by careful cytologic analysis with the aid of fluorescence microspectrography. The analysis of fluorescence spectra is a direct method of identification. The great sensitivity of the fluorescence reaction renders it especially suitable for cytologic analysis. The fluorescence microspectrographic method is undoubtedly not, as regards substances with pronounced fluorescence, to be outdone by any other known method of direct analysis suitable for tissue sections.

The fluorescence microspectrographic method must be employed with great accuracy and discernment. When a physical or chemical method is to be utilized for cytologic analysis, the registration cannot be calculated with the same degree of exactness as in the use of a macro-scale. Therefore, knowledge should have been, or be obtained, by preparative chemical examinations, of the presence in a certain tissue of the substance which is to be localized. Other substances with properties of a confusing similarity at a concentration such as to cause mistakes should, consequently, be eliminated.

As regards fluorescence analysis, an examination of the fluorescence under different conditions will further serve to ensure favourable results. Thus, in the case of riboflavin, the  $p_H$ -dependence of the fluorescence may, for instance, be analyzed and the reversible extinction of the fluorescence by reduction may be determined.

The demonstrated localization of riboflavin in the choroid membrane of the frog's eye repudiates the assumption that it increases the sensitivity of the eye to short-wave light, which causes riboflavin fluorescence in the eye, as maintained by v. ERLER and his collaborators. The riboflavin is screened off from the visual cells by the thick pigment layer in the pigment epithelium. The possibility of small concentrations of free riboflavin in the pigment epithelium and, perhaps, in the visual cells where they would function in this way, cannot on the other hand be excluded, though it has not so far been adequately proved.

The localization of riboflavin and thiamin in the tissues and cells offers indirect support for the distribution of the various ferments of which they form a part, and, accordingly, of the part played by the various cell structures with regard to cellular metabolism.

### Summary.

1. A method of fluorescence microspectrography has been elaborated for the localization of fluorescent substances normally occurring in the tissue cells.

The method has primarily been utilized for cytologic localization of riboflavin and thiamin. An earlier improved method of fluorescence microscopy (SJÖSTRAND 1944) has been employed in this connection.

2. Riboflavin occurs in frogs' eyes localized to the choroid membrane.

3. Thiamin is localized to the myelin sheaths in the peripheral nerves.

To Professor TORBJÖRN CASPERSSON I am greatly indebted for kind interest and valuable advice. The work has been supported by the Therese and Johan Andersson Memorial Foundation, the Cancer Society and by the special Fund of the Medical Nobel prize-section.

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## On the Chemical Evaluation of Digitalis with the Baljet Reaction.

By

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Since BALJET in 1918 showed that a red-orange colour is developed when active cardiac glycosides are mixed with an alkaline picrate solution, the reaction has been extensively used for their quantitative estimation. KNUDSON and DRESBACH (1922) compared the colour of the unknown solution with a strophanthin standard in a colorimeter. Later, LENDLE and SCHMELZER (1935), HAGEMEIER (1939) and NEUMANN (1936) showed that the colour intensity was roughly proportional to the number of molecules containing the unsaturated lactone group.

Recently, BELL and KRANTZ (1945) have modified the Knudson-Dresbach method by the use of an electrophotometer. They found a good agreement between their method and the U S P cat method. However, as already shown by BALJET, the reaction is given also by aglycones and genins. Owing to this fact, it seemed to us improbable that ageing digitalis solutions or digitalis more or less roughly treated during extraction would show such a close parallelism between the colorimetric and the biological assay. In order to investigate the problem, the method was applied to the assay of some mostly old infusions and pills of *digitalis purpurea*.

*Method.* The preparations were made according to the method of BELL and KRANTZ. The extinction was measured in 2 cm absorption cells in a stepphotometer with filter S 50. A standard curve was prepared with a national digitalis standard preparation extracted with al-

cohol. The volumes of the solutions to be tested were chosen so that the extinction ranged between 0.5 and 1. The alcohol concentration in the absorption cell was always kept low and never exceeded 4 per cent. — The biological assay was made on guinea pigs according to the method of KNAFFL-LENTZ. The mean error was always kept below 6.5 per cent of the lethal dose. All values in the table are expressed as potency in percentage of the international digitalis standard powder of 1936.

*Results.* The value obtained from the different digitalis purpurea preparations are summarized in the table. The values from the colorimetric and biological assays of the fresh solutions agree tolerably well. As was expected, the results obtained with the ageing preparations by the colorimetric method did not show the same decrease as the values of the biological assay (Nos. 1, 2, 4, 7, 9). Warming in the thermostat soon caused the infusion to deteriorate, as found in animal experiments, whereas the colorimetric value was unchanged or somewhat increased (6, 11). The same results were obtained with an infusion of the leaves of *digitalis lanata*. The reason of the increase is unknown. It may, however, be mentioned that digitonin, whilst not producing the colour reaction, increases the effect of the glycosides (BIEHLER and RIST (1924)).

We also found small differences between the colorimetric values of the national standard preparations at different times. They were not large enough, however, to affect the conclusions that could be drawn from the material.

### Discussion.

During the preparation of this work, VOS and WELSH (1945) published a report on the Bell and Krantz method with results quite in accordance with ours. It therefore seems that the colorimetric method, even though perfected with the use of an electrophotometer provided with a suitable filter, will give no reliable value for the biological effect of the preparation.

### Summary.

The BELL and KRANTZ modification of the Knudson-Dresbach colorimetric assay for digitalis was investigated, with the use of

solutions diminishing in strength. The colorimetric method gave no adequate conception of this deterioration.

T a b l e  
*giving values for digitalis purpurea preparations.*

	Potency in percentage of international standard	
	Chemical assay	Bioassay (guineapigs)
1) Infusion (1:100) one year old .....	98	37
2) Digifolin, liq. prep. 5 years old .....	32	15
3) Digisolvlin fortior, fresh solution .....	65	85
4) Infusion (1:150), prepared 1943 .....	90	53
5) Infusion (1:150), freshly prepared from the same drug as No 4 .....	118	85
6) No 5 after 22 days at 50° C .....	126	48
7) Pills, prepared 1942, extracted with alcohol from the same drug as No 4 .....	102	64
8) Alcohol extract, freshly prepared (from the same drug as No 4) .....	95	100
9) Infusion (1:100) one year old .....	87	39
10) Infusion (1:100) freshly prepared .....	98	92
11) No 10 after 23 days at 37—50° C .....	133	58

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## Intestinal Pain: An Electrophysiological Investigation on Mesenteric Nerves.

By

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As is well known, WILLIAM HARVEY was the first to observe that an internal organ such as the heart does not possess a sensibility of the same kind as the skin, *i. e.* he discovered that light mechanical stimuli applied to the heart of a conscious man were not recognized by the patient. He therefore stated that the heart was without the sense of touch. Surgeons from LENNANDER in 1901 to our time maintain that the bowel is completely insensitive to mechanical stimuli of any strength provided that the mesentery is not involved. (For full references see "Pain" (1943) and LEWIS (1944).

Using the reflex contraction of the abdominal wall of the cat as a sign of the activity of the nociceptive afferent fibers, LEWIS and KELLGREN (1939) found that these reflexes could be elicited by pinching structures lying in the mesentery of the duodenal loop, as for example the pancreas, but that no such reflexes could be provoked from the bowel itself by injury, by distension or by causing contraction, although it was observed that these stimuli produced a rise of blood pressure in the decapitated cat. Recently T. SJÖSTRAND and C. VON EULER have, however, arrived at a different view. They maintain that pinching the intestine itself without any pulling of the mesentery elicits a distinct response from the abdominal muscles of the cat. Further, they observed that the mesenteric part of the intestine, where the

vessels and the nerves enter the bowel, is more sensitive to nociceptive stimuli than the opposite part. A similar view has also been advanced by MOORE (1938).

TOWER (1933) reported that fast action potentials could be recorded from the posterior roots of the frog only when tension was applied to the mesenteric root while slow impulses could be produced by injurious stimulation of almost any abdominal structures. She also observed that strong stimuli gave rise to large but very slow potential waves.

In the cat as well as in man Pacinian corpuscles are abundant along the mesenteric nerves running to the duodenal loop of the intestine and along the nerves entering the parietal peritoneum. Many investigators, *e. g.* RAMSTRÖM (1906), SHEEHAN (1933), GAMMON and BRONK (1935), have discussed the physiological meaning of this distribution of Pacinian corpuscles without reaching any definite result.

As the cat's mesentery offers very good opportunities of isolating very slender nerve strands suitable for a detailed analysis of the slowly conducted axone potentials, we considered it worth while to make them the object of a closer study.

### Technique and Procedure

All experiments have been performed on cats under chloralose anesthesia (0.05—0.07 g per kg body-weight). The splanchnic nerve preparations were made in a manner previously described by GER-NANDT, LILJESTRAND and ZOTTERMAN (1946). The mesenteric nerves were approached at the root of the mesentery and the nerves were dissected distally as they run along the blood vessels towards the intestine. The dissections were made with the aid of a binocular lens ( $\times 3$ ). The cats were given milk some hours before the operation in order to make the lacteal vessels easily recognizable. These vessels are otherwise hard to discriminate from the nerves. It was found very useful to put a black paper moistened with Ringer under the mesentery, as the white nerve strands with the Pacinian corpuscles as well as the lacteals thus stand out very distinctly. In most preparations the nerve was dissected out for 2 to 3 cm from the mesenteric root. The nerves give off fibers to the Pacinian corpuscles, the majority of which lie alongside the blood vessels, although a few isolated corpuscles can also be seen in the mesentery between the larger vessels. In some preparations the nerve was dissected out as far as its entrance into the intestinal wall. In these cases all branches to the Pacinian corpuscles were cut, and the remaining intact afferent fibers of the preparation thus derived from the intestinal wall only. The intestinal loop was covered with

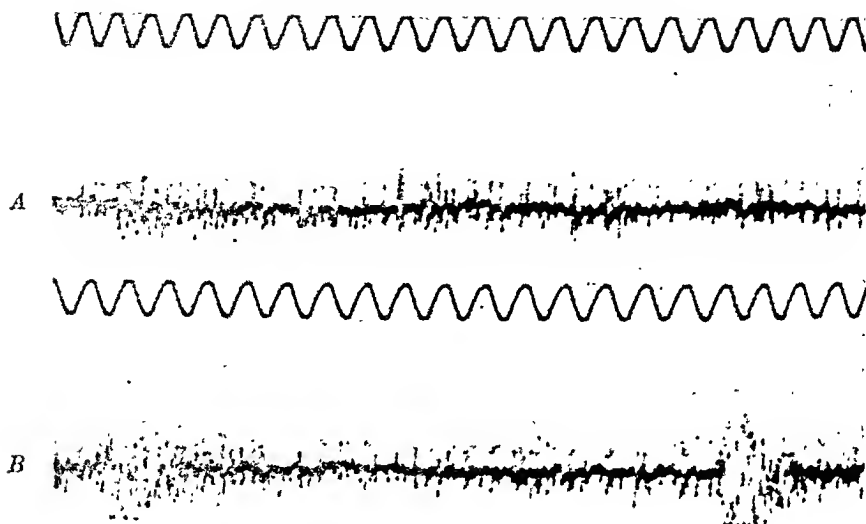


Fig. 1. Cat 2.6 kg. Spontaneous fast spike potentials from the peripheral end of a small branch of the splanchnic nerve: *A*, Control, BP, 110 mm Hg. *B*, After intravenous injection of 5  $\mu$ g adrenaline, BP, 180 mm Hg.

cotton wool soaked in body warm Ringer and the whole animal was kept in a shielded moist chamber at an average temperature of 31° C. When working upon such thin nerves as in these cases one has to be very careful to avoid drying of the nerve. Drying affects particularly the finest fibers, while the largest fibers seem to be more resistant in this respect. Thus it looks as though the thick myelin sheath protects the larger fibers against drying. This difference between the large and the very small fibers may also depend upon the fact that the smaller the fiber, the larger is the surface-to-volume ratio.

The recording of the action potentials was made by means of a capacity-resistance coupled amplifier and a double ray cathode oscillograph previously described (ZOTTERMAN 1936).

## Results.

### The afferent inflow in the splanchnic nerve.

When recording from the splanchnic nerve there is generally a rather frequent number of large afferent spike potentials which seem to flow out partly continuously and partly in distinct volleys synchronously with the arterial pulse (fig. 1). These spike potentials derive from large myelinated fibers which apparently belong to the  $\beta$  group of the class A fibers. When blood pressure

is raised, *e. g.* by adrenaline injections, the volleys of spikes occurring at each pulse-beat may be very largely increased.

These impulses seem to originate from the Pacinian corpuscles situated between the two blades of the mesentery. These organs could be excited by such mechanical stimuli as pulling the intestinal loop and brushing the mesentery. Even a very faint puff of air over the mesentery was sufficient to elicit a rapid volley of impulses. The origin of these impulses was ascertained by recording from mesenteric nerve preparations. These very slender nerves offer much better facilities for a closer analysis of the activity of the different kinds of nerve fibers. From such preparations it was found that large spike potentials were elicited by applying mechanical stimuli close to the course of the nerve in the mesentery, *i. e.* wherever Pacinian corpuscles were found. Brushing very lightly with a very soft pencil over these corpuscles gave an exceedingly massive response of large spike potentials. Even a very gentle puff of air over the mesentery elicited a very frequent volley of these impulses (fig. 2 A).

When these light mechanical stimuli were applied to the intestinal loop, however, it was found that they very often did not elicit any large spike potentials, provided that the stimuli were restricted to the intestinal loop, *i. e.* when stretching or movements of the mesentery were strictly avoided. In some nerve preparations, however, we could elicit large spike potentials by mechanical stimulation of the intestinal wall. In such a case the response obviously originated from a single fiber, the seat of which could be easily localized in the intestine (fig. 3 B). Outside such very well localized spots no large spike potentials could be elicited by light or strong mechanical stimuli, provided that their action was locally restricted.

Strong stimuli, such as pinching or squeezing the intestinal wall were, however, not without effect. On the contrary, such stimuli set up an abundance of slow spike potentials (fig. 2 and 3). These low spikes are apparently very slowly conducted and they must arise from quite thin fibers, most likely belonging to the slowest part of the  $\delta$ -group and very likely also from fibers belonging to the C-class. As these small potentials occurred in groups and thus interfered rather much with each other it was very difficult to measure their frequency. A very localized pressure (fig. 3 B), however, elicited a flow of these small potentials, the frequency of which was estimated at about 600 per second, whereas



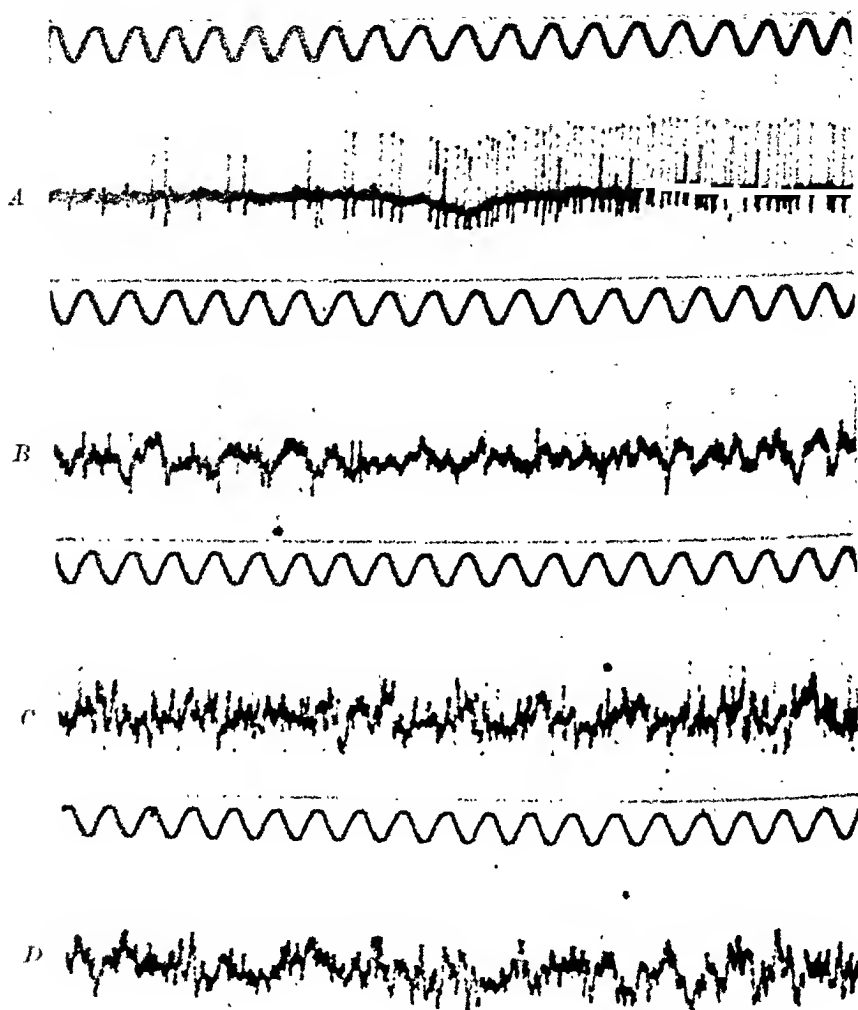


Fig. 2. Cat 2.8 kg. Action potentials from the peripheral end of a mesenteric nerve: *A*, When applying a faint puff of air over the mesentery. *B*, Light pinching of the intestinal loop. *C*, Harder pinching of the intestinal loop. *D* Pressing against the mesenteric nerve in the neighbourhood of the intestinal loop.

the large fiber response consisted of only about 40 impulses per second.

The passing of a strong peristaltic wave was also found to produce a strong response from the small fibers, and a low frequency response from the single large fiber situated in this loop. A stronger

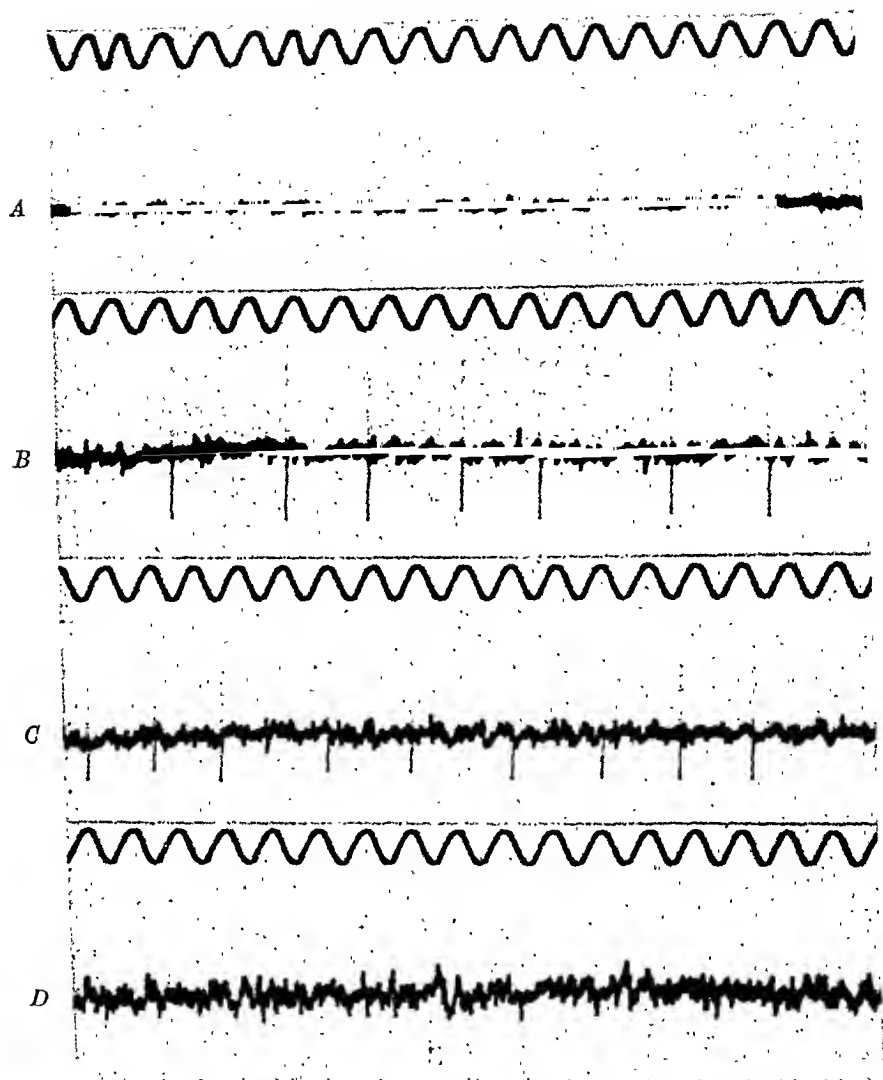


Fig. 3. Cat 2.7 kg. Action potentials from the peripheral end of a mesenteric nerve: *A*, Control. *B*, Pressing upon a very localized spot in the intestinal wall. *C*, After a few  $\mu$ g acetylcholine solution on the intestinal loop, which caused a very strong spastic contraction. *D*, Pinching the intestinal wall just beside the spot mentioned in *B*.

pinching 10 mm caudally from the site of the large fiber gave a very massive response of low potentials deriving entirely from thin fibers (fig. 3 D). A drop of a 1 : 1000 acetylcholine solution upon the intestinal wall, just over the large fiber region, caused a very strong spastic contraction which produced a very definite

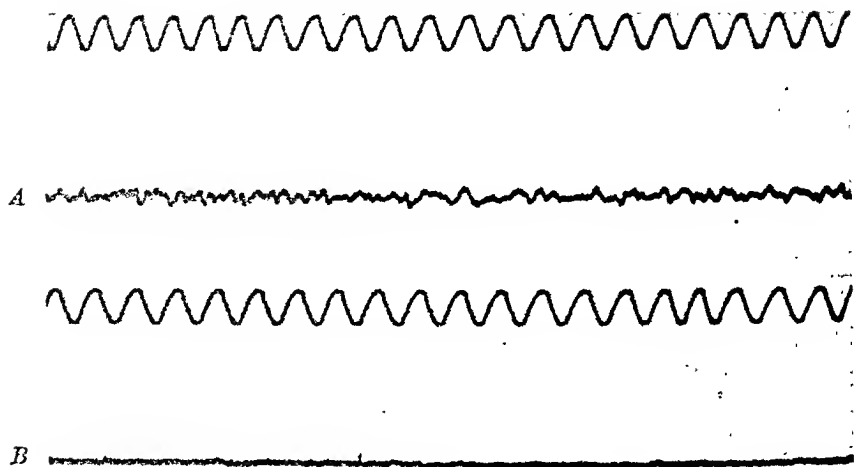


Fig. 4. Cat 2.2 kg. Very slender mesenteric nerve preparation. Peripheral part severed. Action potentials from efferent fibers: *A*, Control. *B*, Nerveligated centrally to the leads.

response in the nerve (fig. 3 C). As will be seen from this record, there is a very distinct outflow of large numbers of very small potentials, and also a very moderate frequency of the large spikes. The ratio between the frequencies of the two groups in this case was estimated as about 20:1.

#### The efferent outflow in the mesenteric nerves.

Records taken from a mesenteric nerve with the leads upon the central end, the peripheral end being severed, showed that there was a steady outflow of centrifugally conducted impulses. Fig. 4 shows these action potentials recorded from a very thin nerve fascicle, about 0.03 mm in diameter, with the leads 2 mm apart. The shape of these potentials is rather irregular and their amplitudes vary a good deal, most likely because of interference between many fibers. Efferent fibers to the region in question are supplied from two sources: a) from the vagus, b) from the sympathetic. The former are preganglionic non-myelinated fibers, the latter postganglionic non-myelinated fibers of very small diameters. If the recorded centrifugal impulses were derived from vagal fibers they would cease when the vagi in the neck were severed. This,

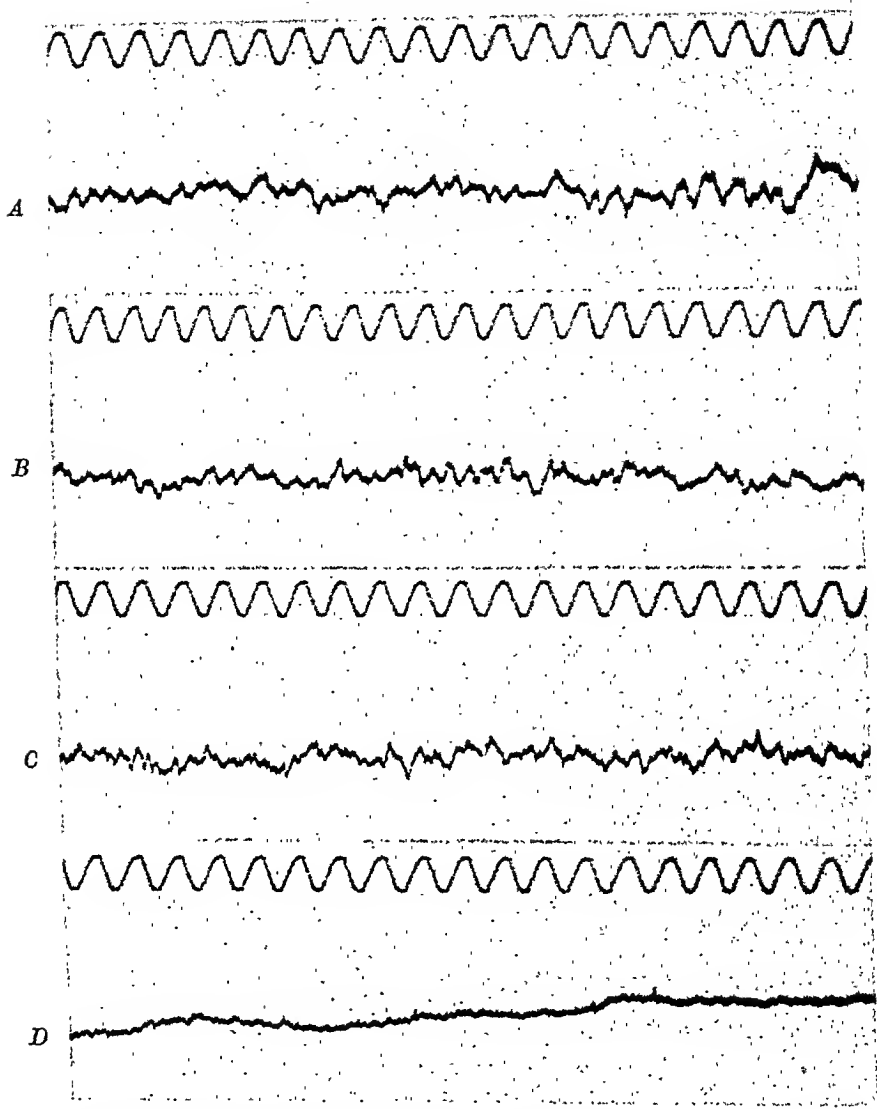


Fig. 5. Cat 2.3 kg. Action potentials from an intact mesenteric nerve: *A*, Control, spontaneous discharge. *B*, After severing both vagi in the neck. *C*, Nerve ligated below the leads. *D*, Nerve ligated above the leads.

however, was not the case as seen in fig. 5 *B*. Severing both vagi did not reduce the potentials to any noticeable degree (fig. 5 *A* and *B*). Thus, we conclude that these potentials are due to an activity of sympathetic postganglionic fibers.

This activity can sometimes be very strong for instance when

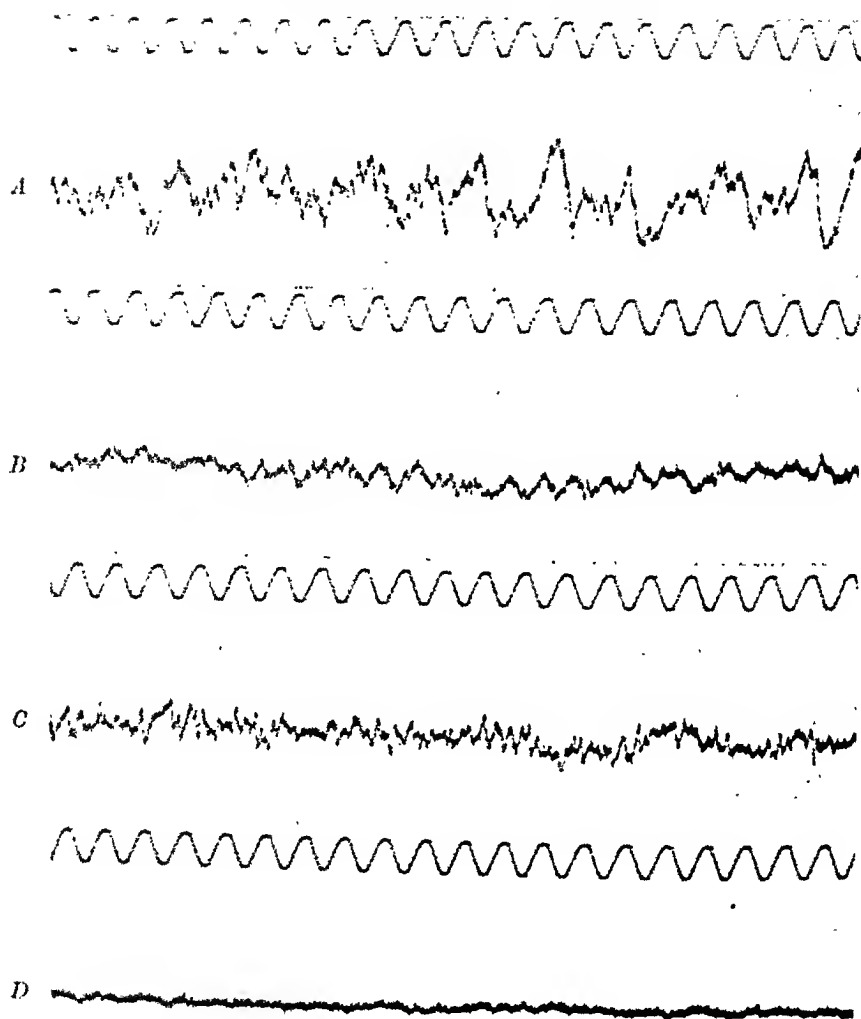


Fig. 6. Cat 3.1 kg. Action potentials from an intact mesenteric nerve: *A*, Spontaneous discharge after previous rough handling of the intestinal loop. *B*, After ligating the nerve above the leads. *C*,  $\delta$ -potentials provoked by pressing upon the intestinal wall. *D*, The nerve ligated below the leads.

the intestines have been roughly handled. In such cases the afferent potentials build up large potential waves (fig. 6 *A*). In this record the nerve was intact, thus allowing the simultaneous recording of afferent as well as efferent impulses. These large

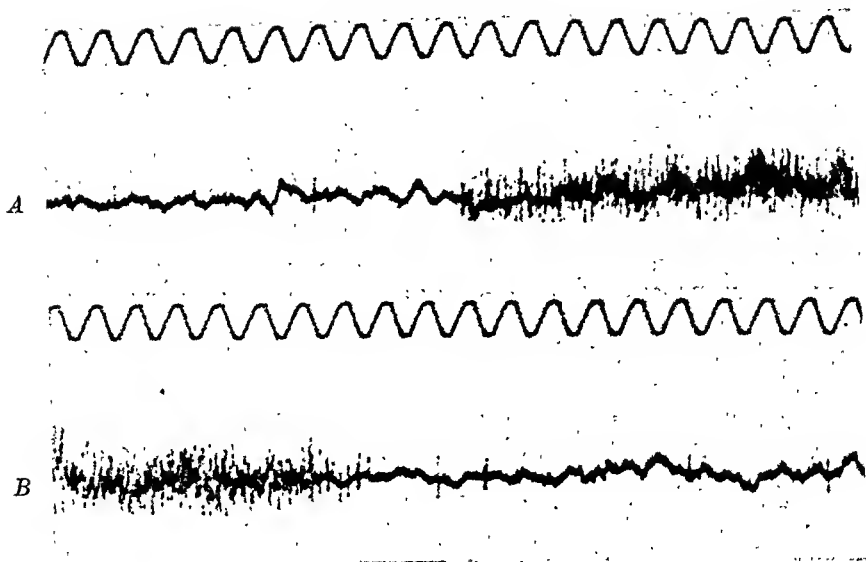


Fig. 7. Cat 2.3 kg. Action potentials from an intact mesenteric nerve: *A*, Shows the beginning of a massive volley of fast spike potentials elicited by a puff of air over the mesentery and *B*, the end of the volley. Note that there is no change in the spontaneous C-fiber activity.

waves ceased entirely upon ligation of the nerve above the leads, which indicates that the waves were built up by efferent fiber activity. The remaining potentials can thus be looked upon as afferent, and were in this case derived from very small fibers only; there were no Pacinian corpuscles seen along the course of this nerve. As will be seen from fig. 6 C, pressing hard or pinching the intestinal wall gave rise to a considerable increase in these small afferent potentials, which seem to derive from small  $\delta$ - and C-fibers.

The very intense efferent sympathetic activity in the record of fig. 6 A was no doubt reflexly set up by an afferent inflow of slow impulses grouped in waves as is seen from the record of fig. 6 B.

These nociceptive impulses conducted in thin fibers are no doubt responsible for the standstill of the intestinal movements observed under these conditions. When leads are placed on the intact nerve the records will show the afferent as well the efferent activity simultaneously, as in fig. 6 A. But it is very difficult to distinguish these activities from each other from the shape of the action potentials, as they are both conducted in very thin fibers. It is thus

impossible to tell to what degree the increased activity in response to pinching is built up by reflexly elicited efferent impulses. It was only the effect of the large afferent fibers upon the efferent outflow which could be tested in this way. When air was blown upon the receptive field of the nerve there followed a very massive inflow of large spikes. It was remarkable, however, that such strong volleys of impulses in large afferent fibers did not provoke any volley of efferent impulses. This is clearly seen from fig. 7, where the spontaneous small fiber activity is the same after as before the strong volley of large spikes.

### Discussion.

From our records it is thus obvious that the walls of the small intestine of the cat are supplied by an abundant mass of fine afferent fibers belonging to the  $\delta$ -group and most likely also to the C-class. In addition, there are a few larger fibers of the  $\beta$ -group. These large fibers in the intestinal wall are very scarce, and large spike potentials are only elicited from a very few localized zones of the intestine. When pressure is exerted the large fiber of the intestine responds very promptly to fairly light touch, and the endings of these large fibers in the intestinal walls have apparently a much lower threshold to mechanical stimulation than the endings of the small fibers, which respond only to more prolonged or stronger mechanical stimuli.

Most excitable to mechanical stimuli are the Pacinian corpuscles situated along the nerve strands running from the root of the mesentery out to the bowel. They are easily excited by a very gentle puff of air, of a strength much less than that necessary to elicit a large fiber response from the intestinal wall, where a visible deformation of the surface was required.

The thin fiber response to pinching etc. had very much the same time course, with a rather long remaining after-discharge, as was recorded from cutaneous nerves when using such stimuli (ZOTTERMAN 1939). The difference between the potential records obtained from mechanical stimulation of the intestinal wall and similar stimulation of the skin is thus that the former gives exceedingly few, if any, large spikes, whilst the latter gives rise to a great number of large and medium sized spikes. The small fiber response to stronger mechanical stimuli is common to both.

Before we proceed further in the analysis of the records described above, we must consider whether all the axone potentials recorded were afferent. As far as the large spikes are concerned it is unquestionable that they derive from true afferent fibers. It is open to question, however, whether all the very low action potentials elicited by such stronger stimuli as pinching the intestinal wall are to be looked upon as afferent in origin. Strong mechanical stimuli might be able to stimulate even efferent fibers in the tissues concerned, which in this case would mean preganglionic vagal fibers and postganglionic sympathetic fibers. The efferent fibers are, however, all non-myelinated, even the vagal fibers as they lose their myelin sheath below the diaphragm. Thus, we cannot exclude the possibility that some of the slowest potentials seen when pinching the bowel may be of efferent origin, but all true  $\delta$ -potentials must be afferent. We know further, from a recent investigation by SJÖSTRAND and VON EULER (1946) that pinching the intestine without any stretching of the mesentery produces reflex contraction of the abdominal muscles as well as a rise in blood pressure, observations which we were able to confirm. Thus there cannot be any doubt that the intestinal wall is supplied by afferent fibers and that the action potentials following upon pinching the intestinal wall are derived in large part, if not entirely, from true afferent fibers.

When strong nociceptive stimuli are applied to the intestinal wall the small potentials often group together building up large waves, as is seen in fig. 2 C and D. Similar large waves from the spinal root of the frog were recorded by Tower (1933), when the abdominal viscera were stimulated and were tentatively interpreted by her as synchronized discharges in efferent sympathetic fibers. As we have recorded from nerves distally from the abdominal ganglia, it seems to us more plausible to interpret the waves in this particular case as being built up by potentials from small afferent fibers, although we cannot completely exclude the possibility that they may to some extent contain antidromic efferent elements also. Such waves were furthermore recorded from cutaneous nerves of the cat by ZOTTERMAN (1939), and were interpreted as built up by slow impulses from the small C-class fibers.

Pacinian corpuscles are found in large numbers in the mesenteric root of the cat and along the mesenteric nerves in the upper part of the small intestine. In man these corpuscles are also found



in the same regions and also in the parietal peritoneum along the branches from the somatic nerves which supply the anterior part of the peritoneum according to RAMSTRÖM (1906).

Pacinian corpuscles are generally believed to serve as organs for pressure reception. Their oval onion-shaped form suggests a function as pressure receptors, and their localization at the joints and along the tendons and more generally in the deeper part of the skin may also be adduced in support of this view. Previous electrophysiological investigations (ADRIAN and ZOTTERMAN 1926, GAMMON and BRONK 1935), as well as our recent experience, have shown that these corpuscles no doubt constitute mechanisms in which mechanical pressure is transformed into afferent nerve impulses. The spontaneous outflow of impulses in large afferent fibers in the splanchnic nerve of the cat derive no doubt from the Pacinian corpuscles, which must be put into action by the slightest intestinal movements as well as by vibration of the body. We thus know that there is a continuous inflow of impulses of varying frequency in large afferent fibers of the splanchnic nerves, which are conducted up to the brain most likely in the posterior columns of the spinal cord. It is quite certain that they do not run in the spino-thalamic tract. What, then, is their physiological significance? As far as we can see, the stimulation of these fibers does not elicit any viscer-enteric reflex, nor does it produce any other noticeable reaction of the animal. The Pacinian corpuscles are thus organs which signal mechanical changes, but they have definitely nothing to do with either nociceptive reactions or with temperature perception, as has been tentatively suggested by some authors. Although we were not able to observe any reactions of the cat to strong stimulation of these Pacinian corpuscles alone, the inflow of these volleys of frequent impulses must of course exert a definite action somewhere in the central nervous system.

It is generally agreed that pulling the bowel and thus stretching the mesenteric root is accompanied with very definite abdominal pain in man. In the cat such a stimulus produces very strong reflex contraction of the abdominal muscles, as well as a rise in the blood pressure. When records are taken from the splanchnic nerve it is found that this stimulus produces a very massive inflow of large spikes, which we conceive as deriving from the Pacinian corpuscles. We are, however, convinced that it is not the activity of these particular afferent fibers, which produces the nociceptive reactions of the animal. These reactions are no doubt

elicited by  $\delta$ - and C-fibers. the electric activity of which can be traced in the records, although it is almost completely masked by the large spikes from the Pacinian nerve fibers.

We know from our experiments upon the mesenteric nerves that all the large fibers are connected to Pacinian corpuscles. It therefore seems very plausible to us that the large fibers of the splanchnic nerve derive from Pacinian corpuscles or similar capsular endings. The splanchnic nerve of the cat contains a good many large efferent fibers of fairly uniform size — their diameters varying from 6 to 10  $\mu$  (fig. 8). All the other myelinated fibers are much thinner, 2 to 3  $\mu$  in diameter. Besides these, there are very numerous non-myelinated fibers. The total number of large myelinated fibers is usually around 300, while the number of small myelinated fibers exceeds 10,000. We do not know how many of these small fibers of  $\delta$ -size are afferent, but they may well amount to about half of the total number.

Now we have stated that no fast action potentials could be elicited from small mesenteric nerves which do not display any Pacinian corpuscles along their peripheral course. Pinching the bowel or the mesentery in such cases gave rise to slow action potentials from  $\delta$ - and C-fibers only. Thus, we conclude that the fast impulses recorded from these nerves derive only from Pacinian corpuscles. As has been stated above, these corpuscles can not be considered as end-organs of nerve fibers which when intensively stimulated could give rise to nociceptive reaction.

Thus we must conclude that the intestine lacks the system of rapid afferent nerve fibers of the skin, but contains afferent fibers of the  $\delta$ -group conducting up to 20 m/sec. and very slow afferent C-fibers, conducting 0.5 to 2 m/sec. Light touching of the intestine does not produce any afferent inflow of impulses provided that the Pacinian corpuscles of the mesentery are not stimulated. Injurious stimuli, however, produce an activity of  $\delta$ - and C-fibers. The response of a mesenteric nerve to injurious stimulation of the intestine is thus very similar to the potential record obtained from a cutaneous nerve when applying radiant heat, which does not produce any mechanical stimulation.

Our experiments upon the mesenteric nerves of the cat thus greatly strengthen the view previously advanced by one of us (ZOTTERMAN 1936, 1939) that nociceptive reactions are not produced by the more rapidly conducting afferent fibers belonging to the  $\alpha$ - or  $\beta$ -group ( $>40$  m/sec). The fast conducting system

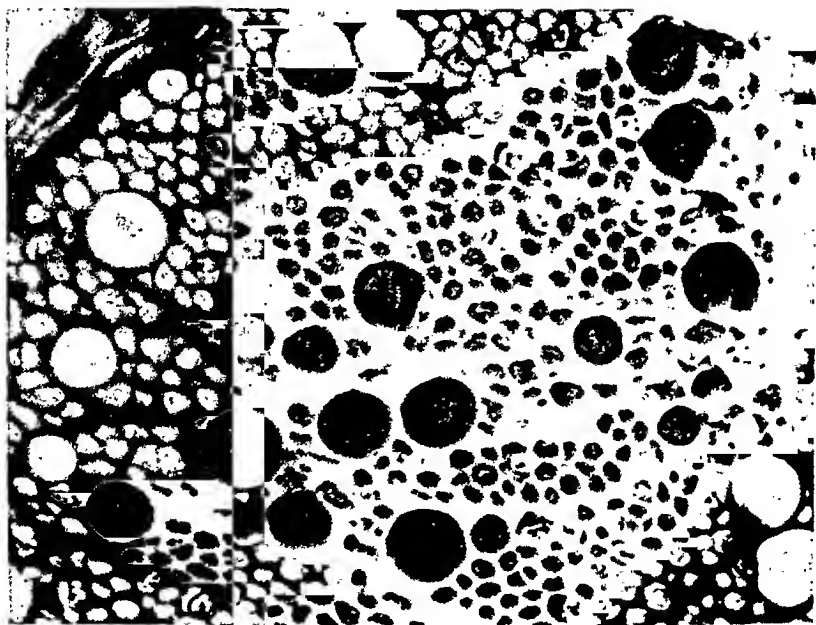


Fig. 8. The upper microphoto shows a transection from the cat's splanchnic nerve, Alsheimer Mann stain modified by B. Rexed,  $\times 180$ . Lower picture tracing from the upper microphoto,  $\times 1000$ .

of pain fibers ("the first pain") is in our opinion an affair of  $\delta$ -fibers while "the second pain" is propagated by C-fibers (ZOTTERMAN 1933, CLARK, HUGHES and GASSER 1935, LEWIS and POCHIN 1938).

### Summary.

Afferent impulses from the abdominal viscera have been recorded from the splanchnic nerve and from fine strands of mesenteric nerves of the cat. The Pacinian corpuscles of the exposed mesentery were found to be extremely sensitive to the most gentle pressure, such as faint puff of air.

The small intestine very rarely receives any large medullated fibres, and light mechanical stimuli such as slight pressure or touch do not give rise to any inflow of impulses. Fast impulses can be elicited only from those parts of the mesentery possessing Pacinian corpuscles.

Injurious stimuli such as pinching applied to the intestinal wall as well as to the mesentery produce  $\delta$ - and C-potentials. In addition to this, intense stimulation gives rise to large potentials waves apparently built up by slow C-potentials.

It is thus concluded that nociceptive reactions from the intestine are not produced by afferent fibers belonging to the  $\alpha$ - or  $\beta$ -group but by  $\delta$ - and C-fibers only.

Support is given for the view that the intestinal wall is supplied by afferent fibers able to produce nociceptive reactions in response to such mechanical stimuli which if applied to the skin, would elicit nociceptive reflexes. The sensibility of the intestine itself is thus to be looked upon as very much the same as that of the skin deprived of its fast-conducting afferent fibers, which respond to vibratory and light mechanical stimuli as well as to stronger pressure.

We are indebted to Dr B. REXED for his kindness in placing microphotos of the cat's splanchnic nerve at our disposal.

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## **A Specific Sympathomimetic Ergone in Adrenergic Nerve Fibres (Sympathin) and its Relations to Adrenaline and Nor-Adrenaline.**

By

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The pioneer work of LOEWI and CANNON and their associates on the mechanism of sympathetic — or rather adrenergic — nerve action has revealed that stimulation of such nerves is accompanied by the liberation of some active principle with sympathomimetic properties. It was primarily assumed that this neurohormonal agent was identical with adrenaline (CANNON and BACQ, 1931) but this view seemed to need further consideration, however, in the light of the findings of CANNON and ROSENBLUETH (1933, 1935) that stimulation of sympathetic nerves elicited remote actions which did not wholly conform with those of adrenaline. In order to reconcile the seemingly conflicting evidence, CANNON and ROSENBLUETH (1933, 1937) elaborated a hypothesis involving the primary liberation from the adrenergic nerve-endings of a mediator substance which should then combine with some constituent within the reacting cells under formation of the final active substances, which, on account of their supposed actions, were termed 'sympathin E (excitatory) and I (inhibitory). The assumption of these authors that the primary mediator should be identical with adrenaline seemed to be supported by the later work of CANNON and LISSÁK (1939) and LISSÁK (1939), who claimed that extracts of various organs and nerves contained a sympathomimetic substance, which conformed in its actions to adrenaline and therefore was different from the sympathins. As

will be shown below certain doubts as to the interpretation of their experimental findings can be advanced. GADDUM and KHAYYAL (1935) showed that stimulation of nerves containing adrenergic fibres liberated a substance which resembled adrenaline in its action on the frog's heart. On stimulation of the postganglionic sympathetic nerves to the perfused rabbit's ear GADDUM and KWIATKOWSKI (1938) found a vasoconstrictor substance in the perfusate which gave the specific test of SHAW (1938) and thus behaved like adrenaline. Recently BÜLBRING (1944) has demonstrated that a sympathomimetic substance is liberated into the perfusion fluid from the superior cervical ganglion on stimulation of the preganglionic nerves. The active fluid gave the fluorescence test of GADDUM and SCHILD (1934) which strengthened the assumption that the active substance was adrenaline, probably derived from the chromaffine cells present in the ganglion. Meanwhile LOEWI (1936) had put forward evidence that the active substance in extracts of frog hearts was identical with adrenaline, which was also supported by the analyses of SHAW (1938) using a sensitive chemical method. On the other hand substances with the properties of the postulated sympathin E or sympathin I do not seem to have been isolated or prepared from tissues or organs in previous work on the sympathomimetic mediators.

Recent work from this laboratory (EULER, 1946 a, b, c, d) has shown, however, that a number of organs contain a blood pressure raising substance which, though it displays several features in common with adrenaline, at the same time shows distinct differences from this substance not only with regard to its action on some test organs but also chemically.

The observation that the nerve-free placenta was the only organ which hitherto was found to be devoid of any activity of this kind suggested that the active substance was connected with nervous structures. The results of CANNON and LISSÁK also provided facultative support to this view. Nervous tissues of various origin were accordingly prepared and extracted, and their action investigated biologically and with colour reactions.

### Methods.

a. *Extraction.* The method of extraction of the organic material used adhered closely to that described in earlier papers (see EULER, 1946 b). In short it consisted in extraction of the minced, fresh tissue

with 2 volumes of acid alcohol, filtering and evaporation of the alcohol. The fatty material present in the aqueous solution was removed with ether. The extracts of this kind (crude extracts) were often tested directly on the blood pressure of the cat but in many instances it was found advantageous to purify the extracts further, especially on account of the presence in the extracts of a factor which lowered the blood pressure. The latter also seemed to be responsible for a stimulatory effect on most smooth muscle organs, which considerably disturbed the biological analysis. It was found possible to remove this factor with Fuller's earth at a slightly acid reaction. After this treatment a pure pressor activity was left in the extracts, though in most cases some loss of active material ensued. A convenient method of testing the specificity of the pressor principle was to add a small amount of iodine solution which quickly destroyed the active substance, leaving any activity due to contaminating substance unchanged.

b. *Test objects.* The extracts were tested on the blood pressure of the cat, on the isolated non-pregnant and pregnant uterus of the cat and the rabbit, the isolated intestine of the same animals and the pupil of the cat. In order to make the blood pressure preparation more sensitive 0.1 mg/kg Gynergen<sup>1</sup> was given i. v. and 8–10 mg/kg cocaine hydrochloride i.m. In some cases it was found advantageous to inject 1 ml 2 per cent novocaine intramedullary to depress vasomotor tone. Gynergen 2–3 mg/kg or dihydro-ergotamine<sup>2</sup> 0.5–0.8 mg/kg was used to inhibit or reverse the pressor action of adrenaline.

The biological effects were compared with those of l-adrenaline, dl-nor-adrenaline (arterenol)<sup>2</sup> and dl, 3 : 4-dihydroxy-nor-ephedrine (corbasil). All compounds were used as hydrochlorides. The dosage of the nor-adrenaline is given in terms of the hydrochloride, the other two as the base. In the experiments on the isolated organs these were suspended in a bath of 30 ml Tyrode's solution bubbled through with 6 per cent CO<sub>2</sub> in O<sub>2</sub>. Temperature 38° C.

c. *Material.* As material for the extracts of adrenergic nerve fibres the thoracic sympathetic trunk of cattle or the splenic artery nerves of cattle or horse has been used. In the majority of the experiments extracts of the latter origin have been used. This material had firstly the advantage of being easily procured in sufficient quantities and in addition yielded extracts of considerable purity and activity. The detailed analysis of the actions presented in this paper were made with this kind of extract, but numerous experiments have shown that the sympathomimetic substance demonstrated in other nerves and in the central nervous system had identical properties in all the mammals used (cattle, horse, cat, rabbit).

Various other nerves were prepared from horse and cattle, such as the vagus, phrenic and splanchnic nerves, several sensory nerves such

<sup>1</sup> Kindly put at my disposal by Messrs. Sandoz, A. G. Basel.

<sup>2</sup> Dr H. Blaschko, Dept. of Pharmacology, Oxford, and Dr. M. L. Tainter, New York, kindly supplied me with generous samples of arterenol, which is gratefully acknowledged.



as the saphenous, fibular and solar nerves, the nerve branches extending cranially from the superior cervical ganglion in the horse, the grey and white thoracic sympathetic rami in the horse, ventral and dorsal (centrally to the ganglion) roots of the calf and some others. Further the spinal cord and various parts of the brain were extracted and tested. In most cases the extracts were tested as crude extracts as well as after purification with Fuller's earth.

## Results.

### I. Biological actions.

#### a. Blood pressure of the cat.

The splenic artery in the horse and in cattle is coated by a plexus of nerves which is largely composed of postganglionic adrenergic sympathetic fibres (BACQ and FREDERICQ, 1935, UTTERBACK, 1944). The plexus can be separated without great difficulty from the arterial wall, thus forming a fairly pure preparation of adrenergic fibres. In the extracts of spleen artery nerves from the horse the biological effect consisted in an almost pure rise in pressure, but also in extracts of the sympathetic chain from cattle the contaminating depressor effect was moderate (Fig. 1). However, in order to perform a proper test of the active pressor substance the extracts were treated with Fuller's earth



Fig. 1. Blood pressure, cat, chloralose.  
 1. 0.01 g thoracic sympathetic chain, cattle  
 2. 1  $\mu$ g dihydroxy-nor-ephedrine  
 3. 0.005 g thoracic symp. chain  
 4. 0.01 g " " "  
 5. 1  $\mu$ g adrenalinine  
 Time 30 seconds.

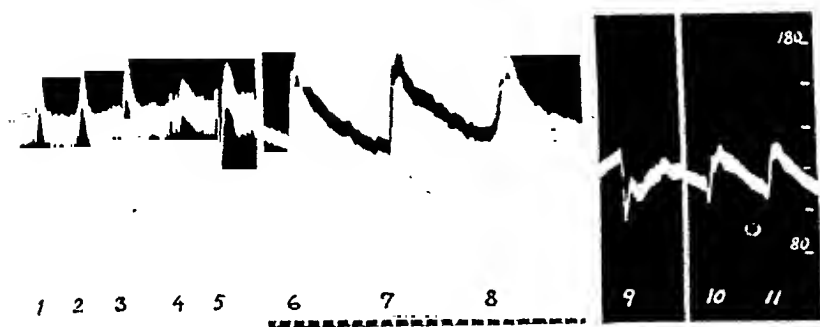


Fig. 2. Blood pressure, cat, chloralose.

1. 0.05 g thoracic sympathetic chain, cattle, extract treated with Fuller's earth.
2. 1  $\mu$ g dihydroxy-nor-ephedrine
3. 0.1 g thoracic symp. chain.
4. 1  $\mu$ g adrenaline
5. 2  $\mu$ g "
- Between 5. and 6. 8 mg cocaine hydrochloride per kg i. m.
6. 1  $\mu$ g dihydroxy-nor-ephedrine
7. 0.08 g thoracic symp. chain
8. 1  $\mu$ g adrenaline
- Between 8. and 9. 3 mg Gynergen/kg i. v.
9. 5  $\mu$ g adrenaline
10. 5  $\mu$ g dihydroxy-nor-ephedrine
11. 0.35 g thoracic symp. chain.

Time 30 seconds.

until no fall in blood pressure preceded the rise (Fig. 2—4). This was found especially important with regard to the effects after dihydro-ergotamine (see below).

On comparison of the effect of the purified extract of spleen artery nerves and adrenaline there appeared a characteristic difference of the same kind as previously described for spleen or heart extracts. Whereas the adrenaline rise before cocaine was mostly preceded by a smaller or greater depressor notch or 'step' with the doses used (about 1  $\mu$ g) no such effect was noted with the active extract (Fig. 2). In the latter case the rise set in rapidly and reached its maximum in a shorter time than the adrenaline rise, when measured from the beginning of the definite rise. In this respect the effect of the extract closely resembled that of nor-adrenaline or dihydroxy-nor-ephedrine, which both produced very similar actions on the blood pressure. Quantitatively dl-nor-adrenaline hydrochloride was about twice as strong as dl-dihydroxy-nor-ephedrine weight for weight, the latter being about as active as l-adrenaline, both the latter calculated as bases.

After treatment of the animal with 8—10 mg of cocaine hydro-

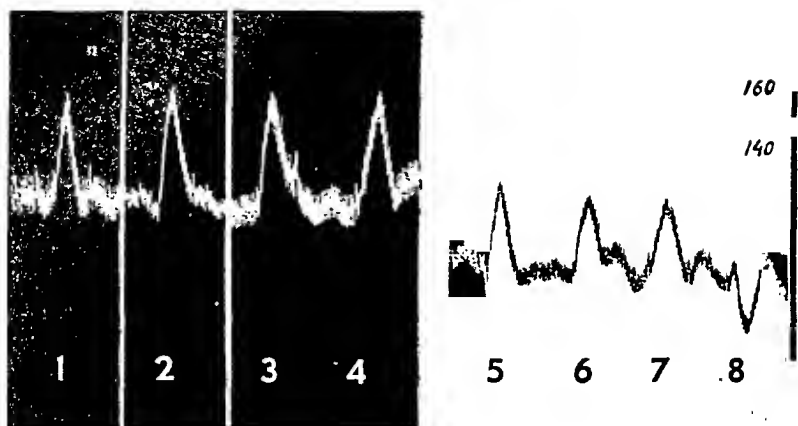


Fig. 3. Blood pressure, cat, chloralose,  
 1. 0.06 g splenic nerves, cattle, extract treated with Fuller's earth.  
 2. 0.5  $\mu$ g nor-adrenaline-HCl  
 3. 1  $\mu$ g dihydroxy-nor-ephedrine  
 4. 1  $\mu$ g adrenaline  
 Between 4. and 5. 0.6 mg dihydro-ergotamine/kg i. v.  
 5. 0.25 g splenic nerves  
 6. 2  $\mu$ g nor-adrenaline-HCl  
 7. 4  $\mu$ g dihydroxy-nor-ephedrine  
 8. 3  $\mu$ g adrenaline.  
 Time 30 seconds.

chloride per kg intramuscularly, a considerable increase in action occurred, not only for adrenaline, nor-adrenaline and dihydroxy-nor-ephedrine but also, and to a very similar extent, for the active extracts (Fig. 2 and 4). Since the enhancement of action by cocaine is characteristic of certain catechol derivatives the observed action supports the assumption that the active substance belongs to this group, which is also borne out by the chemical tests described below.

One of the characteristic features of the remote actions described by CANNON and BACQ, following the stimulation of sympathetic nerves, was the behaviour after a dose of ergotoxine, sufficiently big to reverse the action of adrenaline. In contrast to the action of injected adrenaline the nerve stimulation still produced a rise in blood pressure. The subsidence of the pressor action of spleen or mammalian heart extracts after ergotamine have been previously reported (EULER 1946 b, d). In the present study dihydro-ergotamine was used in some instances besides ergotamine. This new substance possesses the considerable advantage of exerting a sympatholytic action in smaller amounts than

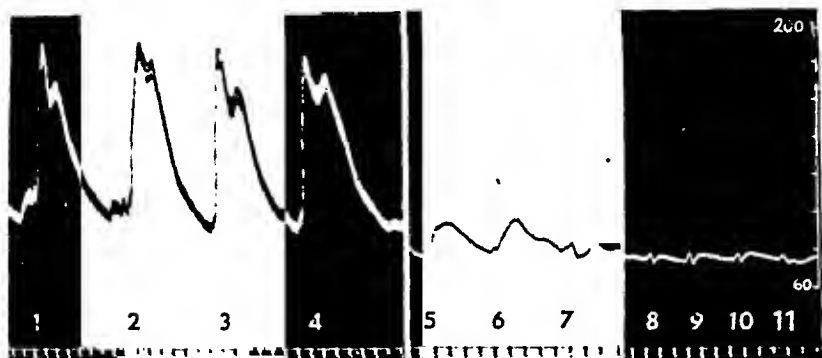


Fig. 4. Blood pressure, cat, chloralose, cocaine-HCl 8 mg/kg i. m.

1. 1  $\mu$ g nor-adrenaline-HCl
  2. 2  $\mu$ g adrenaline
  3. 2  $\mu$ g dihydroxy-nor-ephedrine
  4. 0.1 g splenic nerves, cattle, extract treated with Fuller's earth.
  - Between 4. and 5. 0.8 mg dihydro-ergotamine/kg i. v.
  5. 2  $\mu$ g nor-adrenaline-HCl
  6. 0.2 g splenic nerves
  7. 4  $\mu$ g adrenaline
  - Between 7. and 8. further 0.3 mg dihydro-ergotamine/kg
  8. 3  $\mu$ g dihydroxy-nor-ephedrine
  9. 0.3 g splenic nerves
  10. 3  $\mu$ g nor-adrenaline-HCl
  11. 6  $\mu$ g adrenaline
- Time 30 seconds.

ergotamine or ergotamine and does not greatly interfere with circulation or respiration (ROTHLIN, 1944). After a proper dose of dihydro-ergotamine, 0.5—0.8 mg/kg, the adrenaline effect was reversed, whereas the effect of the active extract, though strongly reduced, still retained some of its pressor action (Fig. 3). It was found essential in these experiments to remove the depressor activity of the extracts with Fuller's earth, since a depressor action, which appeared to be negligible in the ordinary test before dihydro-ergotamine, became more marked after reduction of the pressor response caused by this substance and tended to dominate the picture, thus simulating a reversal. The treatment with Fuller's earth, however, (Fig. 2—4) completely nullified the depressor action before as well as after dihydro-ergotamine or ergotamine.

Thus there was not only a strong resemblance between the action of the extract and that of nor-adrenaline or dihydroxy-nor-ephedrine, but the result clearly excludes adrenaline as the active substance. In no case a reversal of the action of the substances used for comparison or of any active extract sufficiently

purified, was met with after the dose of dihydro-ergotamine used. When there was a complete annulment of the pressor action, as illustrated in Fig. 4, this applied to the extracts as well as to the action of nor-adrenaline or dihydroxy-nor-ephedrine.

*b. Isolated non-pregnant cat's uterus.*

In their study on sympathomimetic amines BARGER and DALE (1910) noted an interesting difference in the action of adrenaline and catechol-ethanol-amine (nor-adrenaline), the latter having a definitely weaker inhibitory action on this test object as compared with adrenaline. A difference of this kind has also been noted by CANNON and BACQ when the effect of stimulation of sympathetic nerves was compared with the effect of injected adrenaline in doses which produced similar effects on the nictitating membrane. Again, when spleen artery nerve extracts were compared with the action of adrenaline in equipressor doses on the isolated non-pregnant uterus preparation of the cat the same kind of difference was noted (Fig. 5 A). The extracts had a distinct inhibitory action, however, which seems of importance for the discussion of its relationship with sympathin E. This inhibitory action was quantitatively of the same order as that of an equipressor amount of

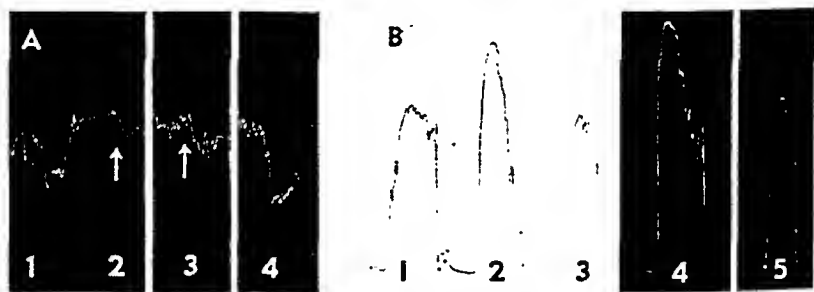


Fig. 5. A. Isolated non-pregnant cat's uterus, suspended in Tyrode's solution, bath volume 30 ml.

1. 5  $\mu$ g adrenaline

2. 2.5  $\mu$ g nor-adrenaline-HCl

3. Extract of splenic nerves, treated with Fuller's earth, equivalent to 1. and 2. on the cat's blood pressure.

4. 5  $\mu$ g adrenaline.

B. Isolated cat's uterus in early pregnancy.

1. 3  $\mu$ g adrenaline

2. 3  $\mu$ g nor-adrenaline

3. 3  $\mu$ g adrenaline

4. Extract of splenic nerves, treated with Fuller's earth, equivalent to 3  $\mu$ g nor-adrenaline on the cat's blood pressure.

5. 3  $\mu$ g adrenaline.

nor-adrenaline, which provides further evidence for the close relationship between the active principle in the extracts and nor-adrenaline.

*c. Isolated pregnant cat's uterus.*

The action of adrenaline on this test object is a purely stimulating one. The same kind of action also occurs with nor-adrenaline though the action of the latter substance is stronger weight for weight (Fig. 5 B). On the other hand adrenaline seemed to be relatively more potent in its stimulating action on this organ than on the blood pressure compared with nor-adrenaline. In every instance, however, there was a complete parallelism between the actions of the latter substance and the purified extracts of adrenergic nerves when equipressor amounts were compared.

*d. Isolated non-pregnant and pregnant rabbit's uterus.*

The isolated non-pregnant rabbit's uterus contracts under the influence of adrenaline and a similar but weaker action is elicited by nor-adrenaline, and by the active extracts, equipressor amounts being compared.

An interesting reaction was observed with a preparation of rabbit's uterus in early pregnancy. This organ reacted with a fall in tone to moderate doses of nor-adrenaline and equipressor amounts of extracts of adrenergic nerves, but with a pure contraction on adrenaline in comparable amounts (Fig. 6). This seems to be the only organ so far examined which answers with a greater inhibitory action on nor-adrenaline than on adrenaline. The complete agreement between the action of nor-adrenaline and the adrenergic nerve ergone therefore receives a special significance. It should be noted too, that dihydroxy-nor-ephedrine also in this respect closely resembles nor-adrenaline in its action.

*e. Isolated rabbit's intestine.*

On this organ principally the same difference was noted between the action of the spleen artery nerves or the thoracic sympathetic trunk and of adrenaline, the latter being considerably more

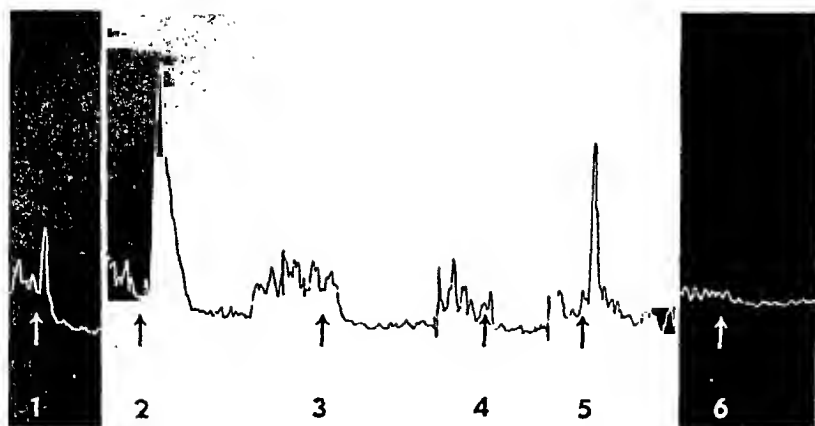


Fig. 6. Isolated rabbit's uterus in early pregnancy, Tyrode's solution.

1. 20  $\mu$ g nor-adrenaline-HCl
2. 20  $\mu$ g adrenaline
3. 40  $\mu$ g dihydroxy-nor-ephedrine
4. Extract of splenic nerves, treated with Fuller's earth, equivalent to 10  $\mu$ g nor-adrenaline on the cat's blood pressure
5. 10  $\mu$ g adrenaline
6. 10  $\mu$ g nor-adrenaline-HCl

potent in its inhibitory action (Fig. 7). The same applies to the cat's intestine.

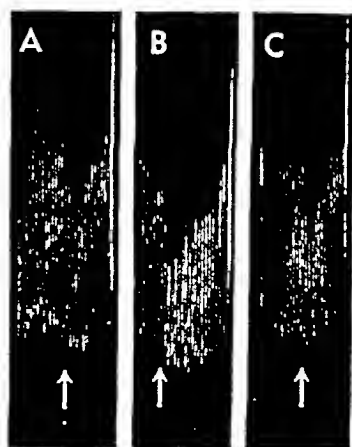


Fig. 7. Isolated rabbit's intestine, Tyrode's solution.

1. 1  $\mu$ g nor-adrenaline-HCl
2. 2  $\mu$ g adrenaline
3. 0.1 g extract of splenic nerves, treated with Fuller's earth, same extract as in Fig. 4. (1—3 same effect on the cat's blood pressure).

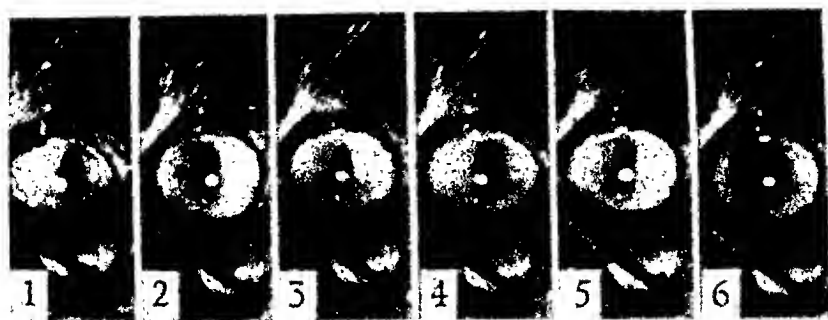


Fig. 8. Cat's eye. Pretreatment with 8 mg cocaine HCl/kg i m. Injections through internal carotid in central direction.

1. Control
2. 2  $\mu$ g nor-adrenaline-HCl
3. Control
4. 0.2 g extract of splenic nerves, treated with Fuller's earth, pressor equivalent with 2  $\mu$ g nor-adrenaline and 4  $\mu$ g adrenaline
5. Control
6. 2  $\mu$ g adrenaline.

#### *f. Cat's pupil.*

One of the arguments for the assumption of CANNON and LISSÄK that the active substance in their extracts was adrenaline was derived from the behaviour of the cat's pupil on injection of the extracts. When, in the present experiments, extracts of the sympathetic trunk or splenic nerves were injected from the external through the internal carotid towards the eye, sensitised by cocaine, a definite widening of the pupil was observed as in their experiments. Quantitatively there was a marked difference from adrenaline in equipressor amounts, however, the latter substance being definitely more potent. (Fig. 8).

## II. Stability.

As previously reported for the active sympathomimetic substance in spleen extracts it showed a fairly good stability in acids but less so in alkali, especially in a purified state (EULER, 1946 b). The reason for the still considerable stability in alkali was explained as being due to the presence of protecting substances.

The purified preparations of splenic nerves or the sympathetic trunk were quickly destroyed by heating to 100° C in normal alkali, but resisted the same treatment with normal acids to a greater extent.



Addition of a small quantity of iodine solution to weakly alkaline or neutral extracts of nerves rapidly destroyed the sympathomimetic activity, leaving the depressor action and the smooth muscle stimulating action, generally occurring in unpurified extracts. In extracts purified with Fuller's earth, where no depressor or smooth muscle stimulating activity was present, a complete inactivation was observed.

### III. Catechol reactions.

All extracts containing sympathomimetic activity gave the characteristic catechol reaction with ferric chloride, the solution turning greenish and, on alalkalization, red. The reaction showed a good quantitative agreement with that of a solution of nor-adrenaline having equally strong biological actions, which is of significance, since a comparison of equally active solutions of adrenaline and nerve extracts on the blood pressure displayed a stronger chemical reaction for adrenaline, which is in conformity with the fact that adrenaline and nor-adrenaline give very similar catechol reactions weight for weight, but are not equally active biologically.

The phospho-tungstic reaction of FOLIN, CANNON and DENIS (1912) was found to be too unreliable in most cases to be of any use for quantitative comparisons, a number of other substances likely to be present in organ extracts, partaking in the reaction.

### IV. Fluorescence reaction.

GADDUM and SCHILD (1934) have shown that the typical green fluorescence in ultra-violet light which appears on addition of strong alkali to an adrenaline solution in the presence of oxygen is very weak in a solution of nor-adrenaline. The same applies to diöxy-nor-ephedrine as announced in a previous communication (EULER, 1946 b). With extracts of the thoracic sympathetic trunk or splenic artery nerves it was not possible to demonstrate with certainty any fluorescence reaction, though an equipressor solution of adrenaline gave a strong reaction, which appears to rule out the possibility of adrenaline being present in significant amounts in the extracts tested.

## V. Distribution of sympathetic ergone in nerves.

It has been shown that the sympathetic ergone appears in alcoholic extracts of various nerves containing adrenergic fibres. In order to gain some more detailed information as to the localisation of the active principle in nervous structures it was firstly investigated whether it occurred in the sympathetic ganglia in amounts comparable to those in the fibres, for instance in the

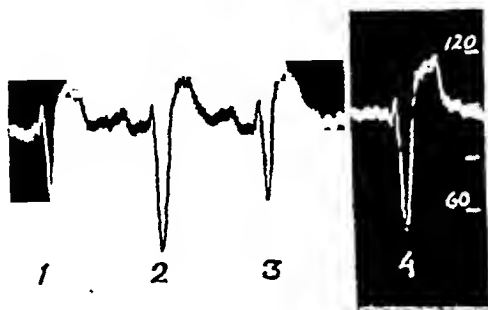


Fig. 9. Blood pressure, cat, chloralose.

1. 0.015 g thoracic sympathetic chain, cattle.
  2. 0.05 g sciatic nerve, cat.
  3. 0.01 g thoracic symp. chain, without ganglia.
  4. 0.05 g " " " " ganglia.
- Time 30 seconds.

splenic nerves. For that reason separate preparations were made of the fibres and of the ganglia of the thoracic sympathetic chain. The tests clearly showed that the content in the fibres far exceeded that in the ganglia, though, of course, numerous fibres were contained in what was prepared as ganglia, isolated by cutting the interganglionic nerve trunk close to the ganglia. The amount of sympathetic ergone in the fibres was roughly 5 times as big as that in the ganglia (Fig. 9). The result seems to merit some interest in view of the recent findings of BÜLBRING (1944) who was able to demonstrate the presence of small amounts of an adrenaline-like substance in sympathetic ganglia in the neck. It appears likely that the adrenaline-like activity found in her experiments is due partly to the existence of adrenergic fibres in the ganglion.

If the activity of our extracts is due to the presence of adrenergic fibres it should be possible to demonstrate different amounts in the white and grey sympathetic rami. This was also the case, when tests were made on such preparations obtained from a horse

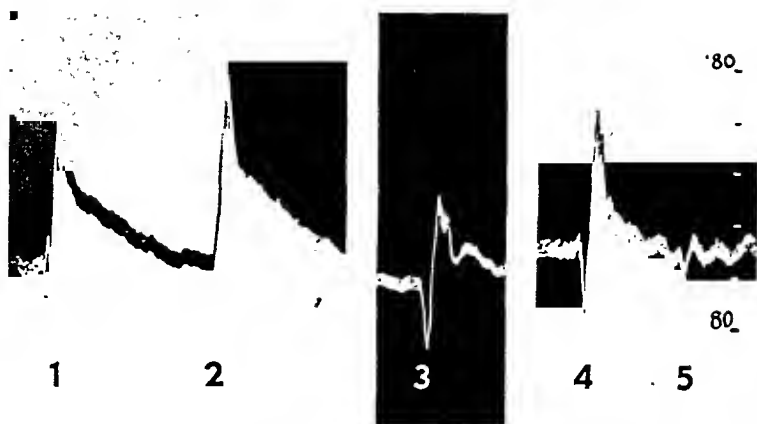


Fig. 10. Blood pressure, cat, chloralose.  
 1. 0.1 g splenic nerves, horse.  
 2. 1  $\mu$ g dihydroxy-nor-ephedrine.  
 3. 0.1 g sensory nerve to the skin of hind leg, horse.  
 4. 0.025 g grey sympathetic rami, horse.  
 5. 0.025 g white sympathetic rami, horse.  
 Time 30 seconds.

(my thanks are due to prof. Palmgren and Dr. Norén of the Veterinary High School for kind facilities offered). Fig. 10 (4 and 5) shows the effect on the blood pressure of the cat of equal amounts of grey and white rami.

Several other kinds of nerves were also prepared and tested mostly only on the blood pressure of the cat. The following table gives approximate figures for the content of sympathomimetic activity in terms of pressor equivalents of adrenaline. Depressor action applies to crude extract.

The highest amounts were accordingly found in the sympathetic nerve preparations, but quite considerable quantities were also demonstrated in extracts of mixed motor and sensory nerves and in sensory nerves.

The presence of the sympathetic ergone in extracts of the vagus nerve, the phrenic nerve and the root fibres of the spinal cord calls for some comment. As to vagus nerves it is known that it contains cardioacceleratory fibres for instance in the cat, which might be taken as physiological evidence for the presence of adrenergic fibres in this nerve. For obvious reasons it has not been possible to demonstrate with certainty the presence of such fibres in the phrenic nerve or in the root fibres of the spinal cord. The amount found in these nerve preparations was not high but

N e r v e	Animal	$\mu$ g adrenaline equivalents per g of nerve in blood pressure test	Depressor action + = weak ++ = moderate +++ = strong
Sympathetic trunk (whole) . . . .	Horse	30	++
"    "    ( " ) . . . .	Cattle	30-100	++
"    "    (fibres) . . . .	"	40	++
"    "    (ganglia) . . . .	"	10	+++
White rami . . . . .	Horse	5-10	++
Grey " . . . . .	"	40	+
Splenic nerves . . . . .	"	20	+
"    " . . . . .	Cattle	20	+
Carotid nerve . . . . .	Horse	10	+
Vagus nerve . . . . .	Horse	5	+++
Phrenic " . . . . .	"	5	++
Mesenteric nerves . . . . .	"	5-10	++
"    " . . . . .	Cattle	5-10	++
Sciatic nerve . . . . .	Cat	5	++
Sensory skin branches to the hind legs . . . . .	Horse	5-10	++
Spinal cord . . . . .	Cattle	2	++
Dorsal roots . . . . .	Calf	2	++
Ventral " . . . . .	"	1.5	+++
Brain			
Cortex . . . . .	Calf	0.5	+
Cerebellum . . . . .	"	0.3	+
Basal ganglia . . . . .	"	0.5	++

sufficient to be significant. Whether the fibres responsible for the content of sympathetic ergone are vasomotor fibres accompanying the nutritive vessels, or appear among the rest of the axones cannot be determined at present.

Only small amounts were found in extracts of various parts of the brain, which had been carefully freed from meninges and superficial vessels. An extract made of vascular plexus and meninges showed, on the other hand, quite a high activity, which indicates that the brain vessels are amply supplied with vasomotor nerves. The latter implication is in harmony with the findings of

BOUCKAERT and JOURDAN (1936). If part of the activity found in brain extracts can be regarded as being due to vasomotor nerves accompanying the vessels it is clear that the nervous structures themselves contain it only sparingly.

As to the presence of the depressor action in the nerve extracts this has been found to be stronger in extracts of the sympathetic chain than in splenic nerves. This is probably connected with the fact that the former contains a considerable number of pre-ganglionic fibres.

#### VI. Effect of severing of splanchnic and splenic nerves on the content of sympathomimetic ergone in spleen extracts.

In three experiments on cats the influence of cutting the splanchnic nerves on the content of sympathomimetic substance in the spleen was studied. The splanchnics on both sides were cut in ether anaesthesia and part of the coeliac ganglion extirpated in two cases. The animals were killed after 14—24 days and the spleen extracted as usual. In these two cases there was a definite reduction of the content of the active substance, though it could still be demonstrated, but in the cat where splanchnicotomia alone was performed no certain effect on the content could be found.

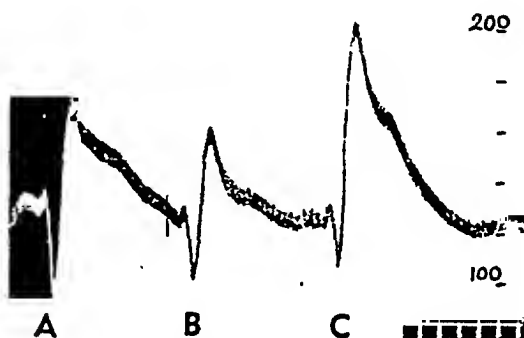


Fig. 11. Blood pressure, cat, chloralose.

1. 0.1 g spleen, cat, two weeks after bilateral splanchnicotomy and extirpation of cranial parts of coeliac ganglia.
  2. 0.1 g spleen, cat, 3 weeks after local silver nitrate treatment of largest spleen artery.
  3. 0.1 g spleen cat, normal (action about equivalent to 0.5  $\mu$ g nor-adrenaline-HCl or 1  $\mu$ g adrenaline).
- Time 30 seconds.

In two other cats the largest splenic artery was prepared free in ether anaesthesia and treated with solid silver nitrate. After 3 weeks the spleen was extracted and the extract tested. The content in these cases was considerably reduced, (Fig. 11) showing that the presence of the active substance is dependent on the splenic peri-arterial nerves. The reason why the effect did not wholly disappear is no doubt to be sought in the fact that the spleen of the cat is supplied with a number of small arteries which convey adrenergic fibres, and possibly also the veins, in addition to the large main branch.

The experiments have thus shown that severance of the splanchnics does not greatly influence the content of the cat's spleen of sympathomimetic substance, but that partial severance of the ganglia and postganglionic fibres reduces the content.

#### VII. Free or bound sympathin in adrenergic nerve fibres?

From the experimental results reported above it seems hard to doubt that the sympathetic ergone, henceforth called sympathin, occurs along the whole length of the adrenergic fibres, thus not only in the 'nerve endings', which are still unsatisfactorily characterized. The question then arises whether the sympathin occurs in a free or in a conjugated form. Some preliminary experiments have been performed in order to gain information on this point, primarily initiated by the previously reported observations on the fixation of sympathin and adrenaline to certain lipids. A binding of this kind was first observed by KENDALL (1942) who found that adrenaline acquired solubility in chloroform in the presence of certain lipids. This observation has been confirmed (EULER, 1946 b) and in addition it was found that sympathin displayed similar properties in this respect. Thus the lipid-ether solution obtained by extraction of the evaporated alcoholic extracts with ether contained some activity which could be extracted by repeated washings with water or an aqueous solution of, for instance, 5 per cent sodium sulphate. Instead of the naturally occurring organ lipids, partly dissolved by the alcohol during the extraction, and subsequently dissolved by ether from the concentrated aqueous extract, brands of commercial phospholipids were dissolved in ether and used as a vehicle. After thorough shaking and separation of the ethereal layer from the aqueous extract, the former had taken up part of the activity which after-

wards could be extracted by an aqueous salt solution. Generally some 2—5 per cent of the total activity of the extract could be transferred in each operation, whereafter a similar amount could be taken up anew and extracted. Thus of 10,000  $\mu$ g adrenaline equivalents in 100 ml extract some 3—500  $\mu$ g were extracted by twice the volume ether containing 50 mg lecithin per ml. This amount could be extracted from the lipid ether solution by repeated (3 times) shaking with 10 ml 5 per cent sodium sulphate solution. Further extractions yielded only minute quantities. The optimal pH of the extract was found to be around pH 4; more acid, neutral or slightly alkaline reactions in the extract yielding smaller amounts of activity. Of the lipids, fats and other lipid-soluble compounds only lecithine and cephaline proved to be of use, other substances such as neutral fats, fatty acids (stearic acid, oleic acid) paraffine oil and bee-wax failing to bring the sympathin in ethereal solution. On the nature of this ether soluble compound of sympathin (and adrenaline) with the phospholipids certain assumptions have been made by BARGER (1930) and by EASSON and STEDMAN (1933) who suggested some ways of chemical linkages between the catechol amines and the phospholipids.

The finding that sympathin may be carried by such lipids in an ether soluble compound suggests a mode of fixation to the lipids of the nerve fibre which possibly is of great physiological significance. If occurring physiologically such a compound would split with sufficient ease to permit a rapid liberation of sympathin in a water-soluble form, judging from the experiments quoted above.

Another possibility would be that the sympathin is fixed to some protein, where some kind of molecular attachment also has been considered in conformity with the suggestions of BARGER. In such a case a high-molecular undialysable compound would be expected to occur. In a number of experiments it was tested whether any significant quantity of sympathomimetic activity was present in an undialysable form. About 100 g of thoracic trunk from cattle was minced and laked with 200 ml water over night in the refrigerator. The clear, slightly rosy fluid was then dialysed against 1 litre of distilled water in the ice chest for 24 hours. It was found that approximately the calculated amount of activity had passed in the dialysate. After renewed dialysis of the content in the cellophane dialyzing bag practically

all of the activity had passed to the dialysate. The experiment demonstrates that, if existing, any high-molecular compound is easily dissociated, allowing the active low molecular group to pass through the membrane. Similar results have previously been reported by LOEWI (1936) and by SCHEINER (1937) who used ultra-filtrates of spleen.

#### VIII. Relations between sympathin and the catechol compounds found in colour tests on organ extracts.

SHAW (1938) has described a method of estimating adrenaline colorimetrically with the use of arsenomolybdic acid, after adsorption of the adrenaline on aluminium hydroxide. He was also able to show that treatment with alkali reinforced the strength of the colour given by adrenaline some 5 times, whereas other catechol compounds such as dihydroxy-nor-ephedrine and nor-adrenaline showed no increase, and, in addition, gave a considerably weaker colour reaction weight for weight. In extracts of various organs and blood from different animals he found the colour increase, due to alkali, except in the suprarenals, only in frog's heart and rabbit's prostate, but not in other organs such as rabbit's heart, liver and others. This is in good keeping with our findings that the active sympathomimetic compound in mammalian heart extracts is not adrenaline (EULER, 1946 d). On the other hand the high ratio of colour increase in rabbit's prostate and frog's heart extracts appears to be of special significance viewing the previous results of EULER (1934) and LOEWI (1936). As shown in the present paper extracts of frog's hearts contain a sympathomimetic substance which does not behave biologically or chemically like that in the mammalian organs but conforms with adrenaline in its action on the blood pressure after dihydro-ergotamine (Fig. 12) and in the fluorescence test.

LOEWI's conclusion that the active substance in frog's heart is identical with adrenaline can thus be confirmed and the evidence for this opinion strengthened.

Using a slight modification of SHAW's method, RAAB in a series of papers has shown that extracts of a variety of tissues contain a colour giving catechol substance, differing from adrenaline in respect of the colour increase due to alkali. He found quite high figures in extracts of a number of organs from rat and man, especially high in the spleen which is in good agreement



with our results. His figures will hardly permit a direct comparison with those found biologically in our previous experiments since other substances than those exerting a pharmacodynamical action probably have been partaking in the colour estimation figures. The statement (RAAB, 1943) that a colour increase greater than 2: 1 indicates pure or almost pure adrenaline or *sympathin* ob-

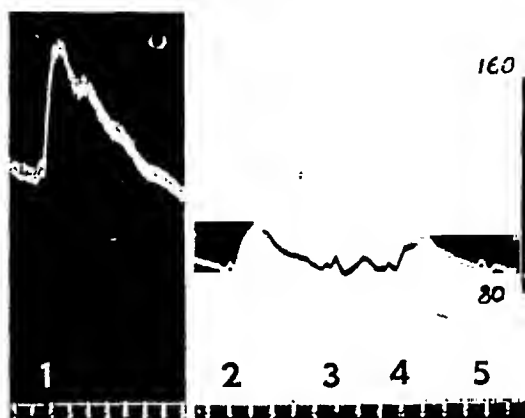


Fig. 12. Blood pressure, cat, chloralose.

1. 0.16 g frog's and toads' hearts (equivalent to 1  $\mu$ g adrenaline)
  - Between 1. and 2. 0.8 mg dihydro-ergotamine/kg
  2. 0.2 mg splenic nerves, cattle
  3. 3  $\mu$ g adrenaline
  4. 1  $\mu$ g nor-adrenaline-HCl
  5. 0.32 g frogs' and toads' hearts.
- Time 30 seconds.

viously needs revision since the latter substance, behaving like nor-adrenaline, does not increase in colour. On the other hand the big activity found in spleen extracts (EULER, 1946 b) as compared with the colour increase ratio found by RAAB for this organ, which had the value of 1, perfectly agrees with our findings that the active substance is not adrenaline.

#### IX. The sympathomimetic ergone in extracts of frog's hearts.

The original work of LOEWI and his associates on the heart stimulating agent liberated from the frog's heart on stimulation of its sympathetic nerves pointed at adrenaline or a nearly related substance as the cause of this action. In later papers (LOEWI, 1936, 1937) evidence was presented for the identification of the

active substance with adrenaline, chiefly based on its behaviour in the fluorescence test of GADDUM and SCHILD. In view of the different behaviour of the active agent in mammalian heart extracts it seemed of interest to repeat the experiments of LOEWI with extracts of frog's hearts. Extracts were prepared in the usual way and tested on the cat's blood pressure and for the fluorescence reaction. As shown by Fig. 12 the extracts produced a clear rise in blood pressure after purification with Fuller's earth, corresponding to some 5  $\mu$ g adrenaline per g. After treatment of the animal with a dose of dihydro-ergotamine, sufficient to inhibit completely the action of adrenaline, the frog heart extract was also ineffective in contrast to nor-adrenaline and splenic nerve extract. This experiment thus definitely points against nor-adrenaline as the active agent and in favour of adrenaline. A comparison of a purified extract of frog's hearts and of adrenaline, having the same action on the cat's blood pressure per unit of volume, as to the fluorescence reaction showed a very similar degree of green fluorescence, which has not been found with extracts of mammalian hearts.

Our results with extracts of frog's hearts therefore fully agree with the opinion of LOEWI that the active substance in this case is adrenaline.

### Comment.

The sum of evidence presented in this report allows the conclusion that the sympathomimetic compound found in extracts of mammalian adrenergic fibres is different from adrenaline. On the other hand the active principle has the properties of a catechol compound, and a detailed study discloses a near relationship with a nor-compound. The assumption that the active substance is in fact the nearest relative to adrenaline, nor-adrenaline or catechol-ethanolamine, is suggestive. To the biological criteria, which support this idea qualitatively as well as quantitatively, may be added the chemical ones, viz. the catechol colour reactions with ferric chloride and the fluorescence tests.

The idea that the specific ergone of the adrenergic system may be nearly related to nor-adrenaline has been expressed previously by several authors though conclusive evidence has been lacking hitherto. Thus BARGER and DALE (1910) were the first to point out the resemblances between the effect of sympathetic stimula-

tion of certain organs and the effect of nor-adrenaline. The discrepancies in action between the action of injected adrenaline and stimulation of the hepatic nerves observed by CANNON and ROSENBLUETH (1933) led BACQ (1934) to suggest that the inhibitory actions caused by sympathetic stimulation were caused by adrenaline whereas the stimulating might be produced by nor-adrenaline. PINKSTON, GREER, BRANNON and BAXTER (1937) and STEHLE and ELLSWORTH (1937) also have considered nor-adrenaline as a possible sympathetic hormone, the former authors on the basis of direct comparison of the effects of this substance with those of adrenaline and hepatic nerve stimulation.

BLASCHKO (1942) has presented chemical evidence for the formation of nor-adrenaline in the body and points out that a demethylation of adrenaline as the source of the postulated nor-adrenaline, believed to be identical with the postulated sympathin E (BACQ), is not needed, but that, instead, adrenaline is formed by N-methylation of the nor-adrenaline.

Obviously this finding gives a strong support to the inference drawn from the experiments described in this paper that the active substance is, in fact, identical with nor-adrenaline.

The observations on the actions of the naturally occurring adrenergic ergone also appear to throw some light on the problem of the two kinds of action of sympathetic stimulation. One of the basic assumptions of CANNON and ROSENBLUETH for the hypothesis of an inhibitory and an excitatory sympathin was that a purely excitatory action could be elicited by stimulation of certain adrenergic nerves. The nerves to the spleen would seem to be a most satisfactory preparation for the study of such an effect. Now it is true that the active ergone in these nerves, as in other adrenergic nerve preparations, exhibits much stronger stimulating actions than inhibitory ones (for instance on the non-pregnant cat's uterus), but the important point appears to be the fact that there is still a definite inhibitory effect on various organs. The adrenergic ergone or nor-adrenaline would therefore be able to elicit most of the inhibitory effects observed on sympathetic nerve stimulation, with the possible exception of the blood vessels, where an inhibitory action is questionable. On the other hand the vasodilator action observed by DALE (1913) on splanchnic nerve stimulation after ergotoxine was rather weak and may be explained either on the basis of antidromic nerve stimulation or on the assumption that adrenaline was released from other

sources than the adrenals, for instance the prostate or the ovaries or paraganglionic tissue.

The experiments of CANNON and ROSENBLUETH give some evidence for the appearance of a factor producing a depressor action after ergotoxine on stimulation of certain sympathetic nerves. This action may be explained by the liberation of small quantities of adrenaline deriving from chromaffine cell groups which have been demonstrated histologically in various places of the body.

From this brief review of the present evidence it seems most likely that the adrenergic transmitter is nor-adrenaline and that sympathetic nerve stimulation on some occasions may liberate small quantities of the methylated product from chromaffine cells in addition, presumably adrenaline. Thus, using the nomenclature of CANNON and ROSENBLUETH, sympathin E would be nor-adrenaline and sympathin I adrenaline, as assumed by BACQ (1934), though he seems to have abandoned this view later (BACQ and FREDERICQ, 1935).

A fact of considerable general biological interest is the demonstration of comparatively large amounts of the sympathomimetic substance in adrenergic nerve trunks, which means that it is not restricted to the nerve endings. This will also explain why extracts of whole spleen contains such large amounts, which, if concentrated to the "nerve endings" would give unlikely high figures.

The adrenergic ergone of the present investigation obviously would have a different meaning than sympathin since this word has been used for adrenaline (CANNON and BACQ) and to depict a substance other than the mediator (CANNON and ROSENBLUETH). On the other hand there is little reason to introduce new names and it is therefore proposed that the specific adrenergic ergone shall be termed sympathin in the meaning of the active transmitter, different from adrenaline.

### Summary.

Extracts have been prepared of various kinds of nerves and their content of sympathomimetic activity determined and the actions analyzed.

The thoracic and lumbar sympathetic chain and the splenic periarterial nerves were especially suitable for preparation of the

active substance and contained some 30—100  $\mu$ g adrenaline equivalents per g as measured on the blood pressure of the cat.

The active substance gives catechol reactions and bears near relations to adrenaline, but differs characteristically from this in the following respects, where a close resemblance to nor-adrenaline was found:

Blood pressure action on the cat after ergotamine or dihydro-ergotamine (Fig. 2—4).

Action on non-pregnant cat's uterus (Fig. 5 A), pregnant rabbit's uterus (Fig. 6) and the isolated intestine of the cat and rabbit (Fig. 7).

Pupil-dilating action (Fig. 8).

Fluorescence test.

The active substance occurs in higher amounts in the grey sympathetic rami than in the white rami (Fig. 10).

The content is fairly high in sensory nerves of the skin but low in sympathetic ganglia, the vagus and phrenic nerves and various parts of the brain.

After degeneration of the main portion of the (post-ganglionic) periarterial splenic nerves the content of the spleen of the active substance is greatly reduced.

The possible physiological significance of the solubility of the active substance in ether in the presence of phospho-lipids is pointed out.

- From the experiments it is inferred that the active substance is the physiological transmitter of adrenergic nerve action in mammals and identical with nor-adrenaline.

Extracts of frogs' hearts contain an active substance with the properties of adrenaline.

It is suggested that the name sympathin should be used for the ergone demonstrated in adrenergic nerves, exerting the actions of nor-adrenaline.

The relationship of the adrenergic ergone to sympathin E and I is discussed.

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## The Influence of Anoxia on the Eye Hand Co-Ordination.

By

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As is known generally the oxygen starvation of the brain produces failure of judgment and inability to carry out or to co-ordinate muscular movements properly. As stated by ARMSTRONG (1943), at about 14,000 ft pressure-altitude and above, there is a progressive deterioration of voluntary muscular control.

In this paper I have tried to find out the influence of oxygen deficiency on the eye hand co-ordination.

### Methods.

The subjects for this study were 1 female and 2 males, 28—31 years old. The experiments were made by means of a Pursuit meter apparatus described by ABRAMSON (1929). The subjects had, by means of a steering-wheel, to fix the hand of an ammeter which made irregular sways. The greater the sways the more current passed through the apparatus and was checked as watt-hours by two ammeters, one for the sways to the left, one for the sways to the right. In table 1 these left and right values are summarized and expressed as the averages of 4—6 experiments.

Before beginning the experiments at low pressures the subjects trained themselves in order to achieve a stable value on the sea-level (about 2 months). Even during the experimental period at low-pressures controls were made on the sea level to ensure that the sea-level values had not changed. The experiments were made in a low-pressure chamber. Before beginning the experiment the subject was exposed to pressure-altitude for 5 minutes then tried to fix the hand of the ammeter for 10 minutes after which samples of alveolar air were taken and finally the oxygen mask put on. After 10 minutes rest at altitudes



Table 1.

Subject	Pressure-altitude m.	Baro- meter mm Hg.	Pursuit m values in wattrhs.		Alveolar CO <sub>2</sub>	Alveolar O <sub>2</sub>
			After 15 min. exposure at alt.	After 10 min. on O <sub>2</sub> at alt.		
L. M.	0	760.0	68.6	—	38.2	97.4
	3,000	525.8	63.7	—	31.3	55.4
	4,000	460.7	63.0	—	29.8	56.9
	5,000	405.1	70.8	69.2	27.2	45.0
	6,000	353.8	95.1	79.4	27.8	39.8
	7,000	307.8	109.5	89.2	26.0	35.5
A. J.	0	760.0	64.8	—	38.5	105.3
	3,000	525.8	55.3	—	33.1	63.4
	4,000	460.7	55.3	—	34.7	60.3
	5,000	405.1	55.3	60.5	27.3	47.6
	6,000	353.8	72.1	71.5	25.2	42.3
	7,000	307.8	88.3	70.8	25.8	34.5
H. R.	0	760.0	73.2	—	34.0	114.8
	3,000	525.8	73.4	—	30.4	69.2
	4,000	460.7	66.0	—	26.6	46.1
	6,000	353.8	134.3	84.8	27.2	43.8
	7,000	307.8	125.2	76.7	24.9	36.6

of 5,000 m and above a new experiment was made with oxygen mask on. The experiments were made in the morning when the subjects were rested after a night's sleep.

### Results and Discussion.

The results attained are shown in table 1. We see that simulated altitudes up to 6,000 m do not influence the co-ordination. (The female subject H. R. did not make experiments at 5,000 m.) At the pressure-altitude of 6,000 m and above a significant deterioration of the co-ordination is seen. At the same altitude the other symptoms of anoxia appeared: anesthetics, euphoria, sleepiness. After administering oxygen these symptoms disappeared and the co-ordination was improved. It is assumed generally that the influence of anoxia is noticeable already at about 4,000 m and that the signs of muscular inco-ordination begin to make their appearance. Although single, familiar movements may be performed about as well as usual, more complicated or unusual ones are clumsily and imperfectly executed, BARD (1942). Considering that the subjects before these experiments had been in training about 2 months we can understand why the inco-ordination appears first at 6,000 m pressure-altitude in this case.

### Summary.

1) By means of Pursuit meter apparatus the influence of anoxia on eye hand co-ordination has been investigated.

2) At pressure-altitudes of 6,000 m and above an appreciable deterioration of co-ordination was found.

The writer is indebted to Ants Juurup for technical assistance.

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## The Influence of Body Temperature on Performances in Swimming.<sup>1</sup>

By

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Numerous investigators have demonstrated that muscular exercise produces a rise in body temperature. This has generally been looked upon as a sign of insufficient temperature regulation. NIELSEN (1938) assumes that the higher body temperature during exercise may benefit the performance of work. He also suggests a way to demonstrate this, *i. e.* by measuring the maximum capacity for work of subjects at different body temperatures.

This has been done by ASMUSSEN and BØJE (1945). They assume that the improvement in work capacity is mainly due to the higher muscle temperature. A higher rectal, *i. e.* blood temperature, allows the local muscle temperature to be maintained at a higher level than would otherwise be possible. Likewise they assert that the passive warming of the organism as well as the active warming through muscular exercise improves the capacity for work.

In this paper I have investigated the influence of body temperature on performances in swimming, which is of some interest considering that the temperature regulation may be severely influenced by the temperature of the water. I have investigated the effects of passive and active warming.

### Methods.

Crawl (50 m and 400 m) and 200 m breast-stroke were used. The rectal temperature was determined by an ordinary maximum thermometer, in some cases where a continuous registration of rectal

<sup>1</sup> The expenses of the work have been defrayed by the E. J. Långmans Foundation.

temperature was necessary a thermocouple was used. Muscle temperatures were measured by means of a thermocouple.

The swimming took place in a 50 m swimming pool, with water temperature 21—22° C. The air temperature in the swimming hall was 23—24° C. The methods for warming have been recorded in the description of the experiments.

The subjects were healthy sportsmen between the ages of 30 and 32. Two of them had been contest swimmers. At the beginning of the experimental period they were untrained. The experiments took place in the morning and the subjects were practically in a fasting condition.

### Experimental Results.

The results from a series of experiments on 3 subjects are averaged. The subjects were: L. M. 31 years, 185 cm, 77.6 kg; A. T. 32 years, 180 cm, 77.5 kg; both former contest swimmers. For the experiments they swam 50 m and 400 m crawl. A. J. 30 years, 183 cm, 85 kg; field-athlete and basket ball player swam for the experiments 50 m crawl and 200 m breast-stroke.

Before swimming the subjects rested (15—20 min.) until their pulse rates reached a stable value. Similarly they did not begin to swim immediately after warming, as they felt dizzy after warming, especially after hot baths, and could hardly swim, but waited here too until the pulse rate was stable (about 10 minutes). The rectal temperature showed in 10 minutes a fall of 0.1—0.2° only, whereas the muscle temperature showed a considerable fall.

The first distance for test-swimming was 50 m crawl. A hot bath was used for warming (40—43° C for 15—18 minutes). The results showed that an increase of rectal temperature by 1.0—1.6° C improves the results by 0.0—2.0 % (averages of 6 experiments). By applying 10 minutes of light gymnastics (jogging), the increase in rectal temperature was 0.4—0.9° C and the improvement in results 0.6—2.2 % (averages of 5 experiments).

As 50 m is a rather short distance and the results may have been influenced by factors beyond our control (for instance the successful or non-successful start or even good control of the swimming-style), longer distances were considered preferable in the subsequent experiments.

As active warming the rather vague "jogging" was replaced by riding a stationary bicycle braked by a simple friction brake. The rate of work was 1,080 mkg/min for 10 minutes. In this and in all subsequent experiments L. M. and A. T. swam 400 m crawl and A. J. 200 m breast-stroke.

Table 1.

*The change in rectal temperature and swimming time after various methods of warming and cooling.*

Subject	Body temperature influenced by:	Number of exp.	Extreme values of rectal temperature		Improvement in time in %
			"Control" cond.	"Warm" cond.	
L. M., A. T., A. J.	Preliminary work	4	37.0°	37.6°	1.4—2.6
L. M., A. T., A. J.	Hot bath	4	36.9°	38.1°	2.1—3.9
L. M., A. J. . . .	Diathermy	3	37.0°	37.7°	1.3—1.9
L. M., A. J. . . .	Cold bath	3	37.2°	(36.5°)	(-3.6—-6.8)

Table 2.

*Percentage improvement in time per 100 m in 400 m and 200 m swimming.*

Subjects	1—100	101—200	201—300	301—400
L. M. . . . .	1.0	0.4	2.5	2.7
A. T. . . . .	1.8	0.4	0.8	1.0
A. J. . . . .	0.8	4.2	—	—

Table 1 shows that here again active warming improves the results by between 1.4 and 2.6 per cent despite the fact that the rectal temperature increases only about 0.6° C. The question as to whether the beneficial effect of the warming is limited to the first part of the distance only or whether it is effective over the entire distance is elucidated in table 2 which shows the improvements of times per 100 m (averages of 4 experiments). We see that every single 100 m in 400 m and 200 m respectively is better in "warm" than in "control" condition.

The results achieved with hot baths (40—43° C in 15—18 minutes) showed a greater increase in rectal temperature when compared with rectal temperatures after bicycle riding, and the times improved more here than in the former experiments.

In fig. 1 the individual values for L. M. in swimming in "control" and "warm" (hot bath) condition are plotted against the successive experimental days. The general trend towards better results during the experimental period is due to the effect of

training. In any case, every result from the "warm" condition is always better than the corresponding "control" result.

In the next experiments the body was heated by means of short wave radio diathermy. Unfortunately, the heating pads at our disposal were not big enough to cover the greater part of the body. Therefore, ordinary heating pads were placed on the hips. It is evident that even this kind of passive warming is able to improve the performances as shown in table 1.

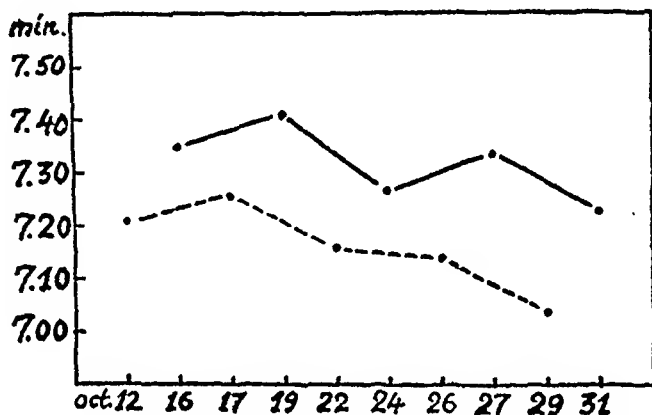


Fig. 1. Performance time for 400 m crawl, plotted against date of experiment. (Subj. L. M.)  
 ..... after hot bath.  
 ..... "control" condition.

The effect of a lower body temperature was investigated in the next experiments. To lower the body temperature the subjects (L. M. and A. J.) stood 12–15 minutes in the swimming pool (21° C). The results achieved are shown in table 1 in which is clearly demonstrated that the lower body temperature before swimming deteriorates the performance.

ASMUSSEN and BØJE (1945) assumed that the muscle temperature plays a more important rôle for the improvement in work capacity than does the rectal temperature.

In table 3 we see the results of experiments made on L. M., swimming 400 m crawl, muscle temperatures taken from M. deltoideus, and A. J. swimming 200 m breast-stroke, muscle temperatures taken from M. triceps and M. rectus femoris (averages of 3 experiments). As usual in this work the temperatures.

Table 3.

*The increase in rectal and muscle temperatures and improvement in time after warming by preliminary work and in turkish bath.*

Subject	Method for warming	Rectal temp.		M. deltoideus		M. triceps		M. rectus f.		Improvement in time in %
		"Control"	"Warm"	"Control"	"Warm"	"Control"	"Warm"	"Control"	"Warm"	
L. M.	Turkish bath	36.7°	37.3°	36.8°	37.5°	—	—	—	—	1.4
„ „	Preliminary work	36.8°	37.2°	37.2°	37.5°	—	—	—	—	2.4
A. J.	Turkish bath	37.0°	37.8°	—	—	36.0°	37.8°	36.5°	38.1°	3.9
„ „	Preliminary work	36.9°	37.3°	—	—	35.9°	37.9°	36.4°	37.9°	3.3

listed in the table are measured immediately before swimming, i. e. 10 minutes after warming. After swimming the muscle temperatures were determined again and they showed an increase in "control" condition by  $-0.1$   $-1.6^{\circ}$  C whereas after swimming in "warm" condition the increase was  $-0.2$   $-0.6^{\circ}$  C. The rectal temperature showed an increase of  $0.1$   $-0.4^{\circ}$  C after swimming both in "control" and "warm" conditions.

As may be seen from table 3 the temperature of M. deltoideus in "warm" condition was only  $0.3^{\circ}$  C higher than in the "control" condition. That raises the question as to whether the higher muscle temperature before swimming has a beneficial effect on the outcome. To solve this problem we let the muscle temperature drop after warming in the turkish bath ( $56^{\circ}$  C 15—18 minutes) until it was on the same level or even  $0.3^{\circ}$  C lower than the corresponding muscle temperature in the "control" condition. We know that the muscle temperature first drops abruptly but later on rather slowly. In our experiments it took about 60 (L. M.) to 80 (A. J.) minutes before the muscle temperature reached the "control" level. During this time the subject sat in a chair with the thermoneedle in the muscle. The rectal temperature remained even after this time about  $0.4^{\circ}$  C higher than the rectal temperature in a "control" condition. The results showed an improvement of 1.2 and 4.3 per cent (averages of 4 experiments).

After every experiment the pulse rate was counted. It showed a constant increase in "warm" condition of 12 (L. M.), 9 (A. T.) and 11 (A. J.) beats per minute as compared with corresponding values after "control" experiments (averages of all experiments).

## Conclusion and Discussion.

It is clearly demonstrated by the experiments on the preceding pages that a high body temperature before swimming facilitates the performance. Likewise it has been demonstrated that a low body temperature has the contrary effect, *i. e.* the results deteriorate. I could record no difference between the active warming of the organism by preliminary work and the passive warming of the organism by hot baths, radio diathermy and turkish baths.

The answer to the question as to whether it is the higher blood temperature or muscle temperature, which is essential for improving the performances in swimming, is that it seems to be the blood temperature.

The ways in which the higher blood temperature may influence the ability to perform work is still open to discussion.

ZUNTZ and LOEWY (1901) state that the higher body temperature is an advantage to the body since it increases the velocity of reactions and so benefits the performance of the work of the organs. BARD (1941) makes the same assumption with the addition that the circulatory and respiration activity is aided by this change.

NIELSEN and HANSEN (1937) found that after heavy preliminary work a subject could take up more oxygen in a final spurt than when the same work had been begun from a resting condition. This effect is ascribed by them to the increased circulation rate reached in the preliminary work. In our case this effect is quite probable as the pulse rate was constantly higher after swimming in "warm" condition as compared with the pulse rate after swimming in "control" condition. Unfortunately there were technical difficulties which prevented us from determining oxygen consumption during and after swimming, which should otherwise have been very desirable.

The problem of the influence of higher body temperature on blood sugar content is quite controversial. Authors such as NOEL PATON (1894), SENATOR (1909), ROLLY and OPPERMAN (1913), WEYL (1929), RAFFERTY and MAC LACHLAN (1943) state that an increase in body temperature calls forth a rise in blood sugar content.

The opposite is shown by the experiments of LEPINE and BOULUD (1910) who found no changes in blood sugar content by increased body temperature. SCHWARZ and KASPAR (1927) sum-



marize their results by stating that the increase in blood sugar content is by no means due to changes in body temperature and that it seems rather to be a symptom of increased metabolic processes. CHRISTENSEN (1931) found an increase of blood sugar content after muscular work had ceased, but a correlation between body temperature and blood sugar content is denied.

When considering the effects of higher body temperature on hormonal processes the work of SAITO (1928) is worth mentioning. He found that by heating dogs until their rectal temperature exceeded about  $41^{\circ}\text{C}$  the velocity with which epinephrine was liberated increased notably, *i. e.* about seven times that during the rest spells. The after-action is stated as "fairly-long". GELLHORN and FELDMAN (1943) assert that heat acts on both vago-insulin and sympathetico-adrenal systems but the predominant effect is on the former.

Of special interest for this discussion is the work of BARCROFT and KING (1909) who have demonstrated that oxyhaemoglobin is dissociated faster and more completely when the temperature of blood and muscles is increased. The same effect accounts for the dissociation of myoglobin, although the temperature dependency is somewhat smaller than in haemoglobin, as was stated by THEORELL (1934).

I conclude that the higher blood temperature before swimming improves the results. It seems to be quite probable that this beneficial effect is due to the increase in the velocity of reactions (circulatory, metabolic and hormonal), but I am not in position to demonstrate it more comprehensively. The muscle temperature increases after swimming but the higher muscle temperature before swimming shows no special effect on the outcome.

### Summary.

The effects of the higher body temperature on performances in swimming have been investigated.

The experiments showed that:

- 1) A given distance could be swum in a shorter time when the organism was warmed previously.
- 2) Both — active warming by preliminary work and passive warming by hot baths, radio diathermy and turkish baths — had the same beneficial effect.

- 3) The increased rectal, *i. e.* blood temperature, before swimming seemed to be more essential for improved results than the increased muscle temperature.
- 4) The duration of the influence of warming was at least 60—80 minutes.
- 5) It is quite probable that the beneficial effect of higher body temperature is due to the increase in the velocity of reactions.

The writer is indebted to Ants Juurup for technical assistance.

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## The Determination of Melting Points in Human Fats.

By

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During an investigation of human fats considerable technical difficulties appeared in the determination of the melting points. When the common methods were used, the results were in many cases not reproducible within several degrees centigrade. In this paper the physical properties of the fats will be the main subject discussed — technical arrangements for heating, measuring of temperature etc. will only be treated briefly.

The melting point of a *pure* substance is the temperature at which the substance can exist simultaneously as a solid and as a liquid.

Natural fats are never pure substances, they are mixtures of different triglycerides, often also containing free fatty acids and other lipid substances. It is very improbable that the components in any fat are present in just those proportions which make up an eutectic mixture. Disregarding this special case, which will perhaps never occur, the natural fats will have a certain "melting range" instead of a "melting point". The term "melting point" of fats may be used as a designation for the upper limit of the melting range, *i. e.* the lowest temperature at which all the fat is liquid. Correspondingly, at the lower limit of the melting range all the fat is solid. Between these two end points liquid fat and solid fat are present simultaneously.

A large number of methods have been developed for the practical determination of the "melting point", which means the

upper limit of the melting range or a point near this. In most fats this point is relatively easily determined. The methods described in laboratory manuals etc. will most frequently appear to be satisfactory. The "melting point" can usually be reproduced within some tenths of a degree.

The "melting point" is defined in different ways according to the method used. It may be *e. g.* the temperature at which the fat sample, by heating becomes totally clear and transparent (clear point, point of complete fusion), or it may be the temperature at which a droplet of fat in a capillary tube begins to move under the influence of the force of gravity or another force (*e. g.* a certain water pressure), or it may be the temperature at which the fat which adheres to a cold thermometer, dipped into a melted fat, assembles to a drop and drips off. Numerous methods using these principles have been developed and described. It must be pointed out that the first principle, the use of the point of final melting, is the real upper limit of the melting range. Other, empirical, methods, which use the relatively rapid change in viscosity near this point, will give a "melting point" that in most cases will be somewhat lower.

When the writer tried to apply on human fats the methods, which are characterized by changes in the viscosity of the fat, it appeared that the variations in the results, when making parallel determination on one and the same sample, were always so large that no constant value could be reached. The variations were often several degrees, in many cases even more than five.

The "clear point" gave in some cases good and reproducible results, but in many samples the melting point reached was very sensitive to variations in the heating velocity and the time the samples had been cooled. In others it was difficult to observe the point when the sample was completely transparent. A very vague opacity remained through a range of several degrees, and it was often difficult to observe the point when it had disappeared completely.

It was of interest to know whether the melting point should be fixed as the temperature at which only a hardly visible opacity was left, or as the temperature several degrees higher where even this minute trace of dimness had gone. In order to decide this question it was of interest to follow more exactly and quantitatively the changes during the melting process.

### The Dilatometric Method.

The best method for following the melting process quantitatively is to observe the changes in volume which appear during the change from one state to another. HOFGAARD (1938) developed a method which permits a quantitative dilatometry of fats, which, better than other dilatometric methods, permits an approximate calculation of the proportions of the fat which are present as solid and as liquid respectively at any point of the melting range.

The dilatometer and the use of it is described by HOFGAARD (1938) and will not be treated here. HOFGAARD always calculated the results as the specific volume of the fat, and the values were plotted in a diagram against the corresponding temperatures.

The use of the method is most easily understood if we regard the schematic diagram in fig. 1.

The curve A—B—C—D represents the specific volume-temperature diagram of a certain fat. At the low temperature,  $T_A$ , all the fat is solid. The fat, like other substances, expands by a rise in temperature, and the expansion is proportional to the temperature increase, *i. e.* the part A—B of the curve is a straight line. At the point B the fat begins to melt: The curve makes a bend, and the part of the curve which now follows, B—C, shows the melting range. At C all the fat is fluid, and from this point on the curve is once more linear (C—D), representing the expansion of the fluid phase. The part B—C needs not be linear, its form depends upon the components of the mixture.

The temperature range  $T_B$ — $T_C$  represents the melting range of the fat in question. A pure fatty acid melts at a constant temperature, and if the melting point is  $T_C$  the dilatometric diagram will have the course A—B—C'—C—D. At temperatures lower than  $T_C$  all the fatty acid is solid, at higher temperatures it is all fluid.

The proportions of solid and fluid phase may be approximately calculated for any point of the curve, because different triglycerides have not very different expansion coefficients. (The more exact treatment, which pays attention to differences in the expansion coefficients, is given by HOFGAARD. For the present reasonings this is not necessary.)

A curve representing the expansion (specific volume) of the solid fraction above the point B, where melting begins, is represented by the dotted curve B—C', and the specific volume of the

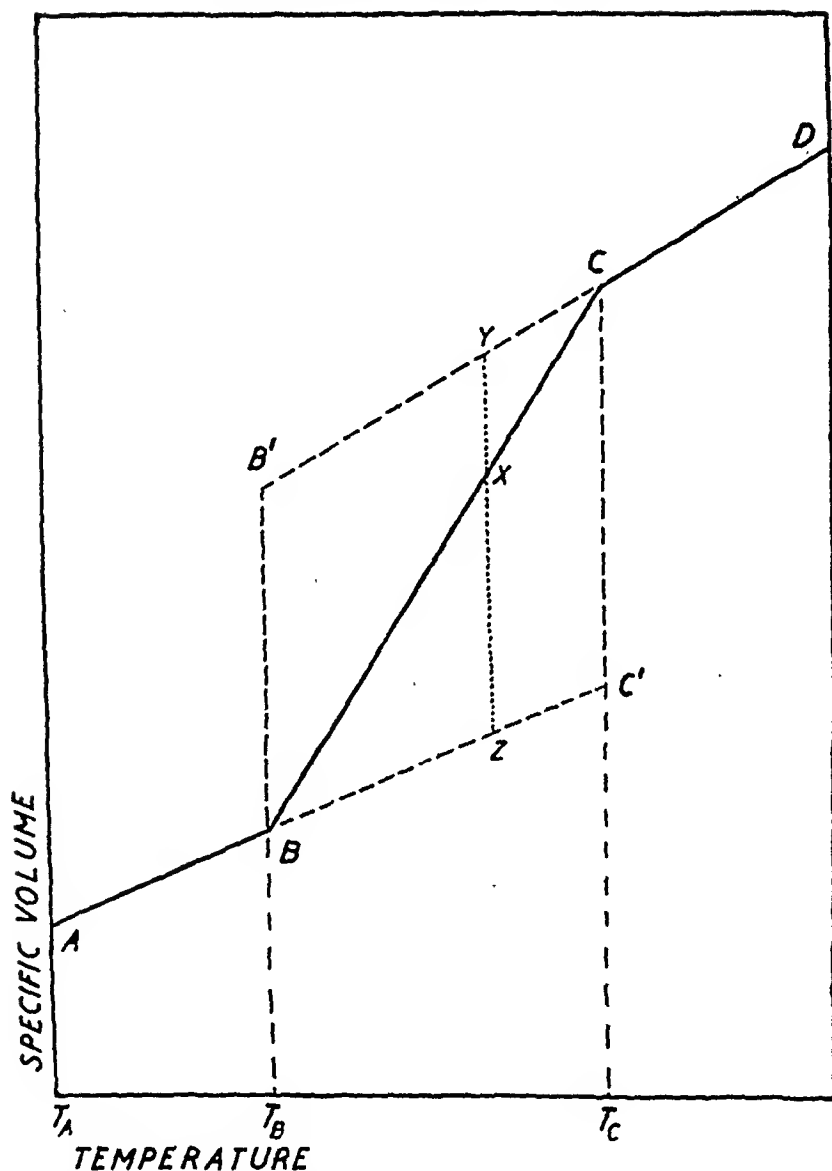


Fig. 1. Schematic diagram illustrating the relation between volume and temperature in fats. A—B gives the dilatation of the solid fat, B—C the melting range, and C—D the dilatation of the fluid fat.

fluid fraction below C is represented by the dotted curve B'—C. For a point, x, in the curve, the ordinate x—y represents the solid fraction, the ordinate x—z the fluid fraction.

A fat which consists of glycerides of not very different nature *e. g.* of stearic and palmitic glycerides, will have a narrow melting range. Fats which in addition to glycerides of high melting point also contain a considerable quantity of low melting components will generally show a wide melting range.

If the line B—C meets the straight line B'—C—D at a relative large angle, we see that even at a temperature very near to the  $T_C$  there is a relative large proportion of solid phase. If B—C and B'—C—D form a very small angle the proportion of solid phase is very small even at some distance below  $T_C$ . In the case first mentioned, which is found *e. g.* in coconut oil, the top end of the melting range is easily determined by the "clear melting point", there are no difficulties in observing the melting of the solid phase, the sample turns from opacity to absolute clearness within some tenths of a degree. The other possible "constants", viz. "softening point", "slipping point" etc., which depend upon a certain low viscosity of the fat, are found near to the clear melting point and are relatively well defined.

On the contrary, when only a small amount of solid phase is present even at some distance below the  $T_C$ , it is much more difficult to observe the point at which the last, hardly visible, opacity disappears. Even at several degrees below the  $T_C$  the fraction of solid phase is so small that the fat can form drops or move in a capillary under the influence of gravity. Further, the change in viscosity per degree is small, and consequently the position of "constants" that depend upon the viscosity is badly fixed.

In these relations we find the explanation of the impossibility to determine "melting points", dropping points, etc. in human fats, and the difficulty to observe the exact clear melting point.

The diagram in fig. 2 gives an example which demonstrates these properties in a sample of human fat. The diagram shows the point for total transformation to fluid phase ( $T_C$ ) at ca 36.5° C. The clear point was 36.4° C when determined according to the technique described below.

Further the diagram shows that near the top end of the melting range very small fractions are present as solid phase. At 6° C below the upper limit of the melting range about 3 per cent of the total fat is present as solid. For comparison can be mentioned that in coconut oil 3 per cent are present as solid at about 0.3° C below the  $T_C$  point.

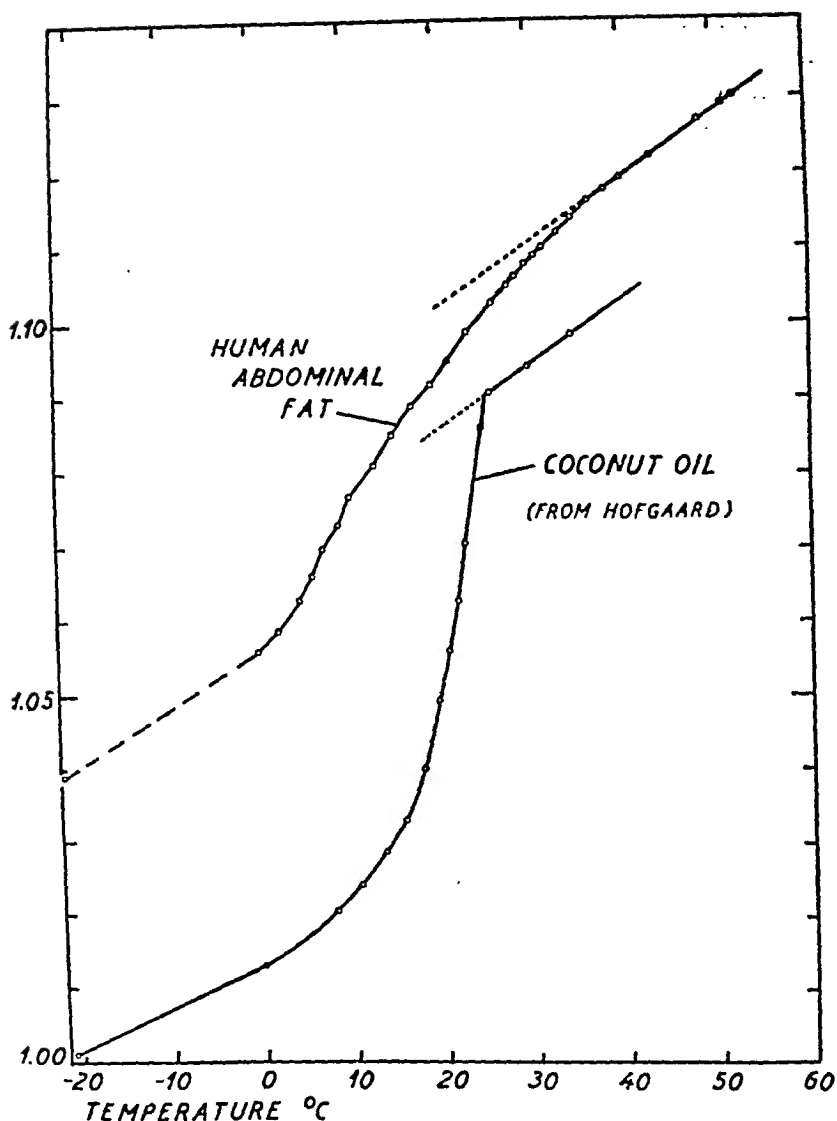


Fig. 2. Dilatometric curve of a sample of human fat. For comparison a curve for coconut oil is quoted from HOFGAARD (1938):

In all the samples of human fats investigated in this way such good agreement was found between the upper limit of the melting range and the clear melting point when this last value had been determined according to the authors method. The difference was always less than  $0.5^{\circ}\text{C}$ . In this way it was demonstrated that the



"clear melting point" observed is really identical with the thermodynamically fixed upper limit of the melting range.

The dilatometric method is unfortunately very cumbersome and too complicated for being commonly used in determinations of melting points of fats. The method requires too much effort and time.

The diagram in fig. 2 is incomplete in the lower part. There are two reasons for this. The laboratory had no equipment for arranging thermostates at temperatures much below  $0^{\circ}\text{C}$ . The low temperature of  $-21^{\circ}\text{C}$  was produced by mixing salt and ice in a big Dewar bottle, here the temperature kept constant for three days. Another reason for the incompleteness of the lower parts of the curve is the tendency shown by the fats to form instable modifications. During the rapid cooling of the dilatometer the fats solidify in unstable modifications, which only slowly turn into the stable ones. A constant volume at a given temperature is not reached before the thermodynamic equilibrium at the temperature in question is reached, and at the lower temperatures this may require a very long time. Considerable contractions will often appear in the first days after a rise in temperature. This is due mainly to unstable modifications, which expand by "melting" and contract again when recrystallizing in a stable form. During the experiment the dilatometer must be kept at each temperature for at least one or two days, and if definite alterations have appeared it must be kept at the temperature until the alterations are inconsiderable. If the alterations are only small it may be regarded as legitimate to raise the temperature even if an absolutely constant volume is not reached. The isothermic "after-contractions" often necessitate a very long time for completing a single experiment. The experiment given in fig. 2 required about 3 months. As an example can be mentioned that the sample showed a contraction at  $10.60^{\circ}\text{C}$  which had not ceased after 20 days. The writer had not sufficient patience to wait any longer, especially since the curve above  $30^{\circ}\text{C}$  was of most interest. At the temperatures near the top of the melting range the allotrope transformations go much faster, and are not a hindrance to the relatively rapid performance of the dilatometric experiments.

## The Determination of the "Clear Point".

In the following the technique developed for determination of the clear melting point of human fats will be described. Some details of the melting process will be discussed, details which are of importance in relation to the tendency the fats have to attain only slowly the most stable condition at the temperature in question. It is of importance to know these properties and to make clear their influence upon the melting point determinations.

### Technique.

The fats for melting point determinations were obtained from relatively small tissue samples. About 1 gram (or more) of adipose tissue was cut finely and ground with quartz sand. It was mixed with water in a centrifuge tube, heated in a water bath, and then centrifuged hot. The separated fat was pipetted into another centrifuge tube and after adding some water-free sodium sulfate it was centrifuged once more. The pure fat was sucked into a capillary of about 2 mm diameter, which had been drawn out into a fine tip. The tip was sealed by touching a gas flame.

The fat was cooled by putting the capillary into a mixture of alcohol and  $\text{CO}_2$ -ice (about  $-70^\circ \text{C}$ ). When a number of tubes had been prepared they were, still left in the cooling mixture, put into the ice-chest at ca  $-5^\circ \text{C}$ . Here the samples gradually attained the temperature of the ice chest, where they should remain for at least one day.

The melting point determination was carried out in a beaker filled with water of  $0^\circ \text{C}$ . The melting point tubes were attached to the beaker by means of some suitable adhesive (plastiline). The water was mixed by a stream of large air bubbles, this is much more convenient than stirring by hand. The water was heated slowly by a micro gas burner — near the melting point very slowly, not more than  $0.5^\circ \text{C}$  per minute.

Some of the samples of human fats had melting points near  $0^\circ \text{C}$ . Their melting points were determined in an alcohol bath cooled with  $\text{CO}_2$ -ice. Owing to the low specific heat of alcohol, the bath attained the temperature of the laboratory air so rapidly that no other heating device was necessary.

The difficulties in observing the small changes in the transparency of the samples near the melting point was overcome by strong side illumination against a dark background.

As demonstrated above the point where the last traces of visible opacity disappear from the sample is the real "melting point" of the fat and identical with the upper limit of the melting range of the fat in question.

The procedure described takes into consideration some relations which ought to be treated in some detail.

A given sample of melted fat can be regarded as a solution of high melting glycerides in low melting glycerides. When the mixture is slowly cooled, a certain point will be reached where the solution is saturated in respect to the high melting fraction. A further cooling will bring about a separation of a solid phase. When the cooling is continued, the crystals grow larger, other components separate, and at last the total fat has solidified as heterogeneous crystal masses.

If a sample cooled in this way is heated again at the velocity commonly used in melting point determinations (continuous heating, about  $\frac{1}{2}^{\circ}\text{C}$  per minute), we will have the following condition near the top end of the melting range. The fluid phase is nearly saturated with the solid phase, which dissolves in the fluid more and more during the heating. A certain rise in temperature gives a certain increase in the unsaturation of the fluid in respect to the solid. The crystals dissolve, but because they are large and have a relatively small surface the procedure is very slow. The heating is continuous, and before the large crystals have dissolved totally, the temperature may have risen several degrees. In the most extreme case the crystals do not dissolve at all, and the sample becomes transparent only when the crystals themselves melt. In this case we observe the melting point of the primarily separated crystals as the melting point of the sample.

If the sample is cooled very rapidly the fats separate as a micro-crystalline mass. During the heating, the solid particles will readily dissolve in the fluid until the equilibrium at the temperature in question is reached. Therefore, near the top of the melting range, the solid microcrystals will in a very short time dissolve in the fluid, the real equilibrium is reached almost simultaneously with the rise in the temperature, and at the top end of the melting range the correct melting point is observed.

In these relations we find the explanation of the necessity of a rapid cooling of the samples. In most methods for melting point determinations a cooling to  $0^{\circ}\text{C}$  is prescribed. This is in no way sufficient for samples of human fats. They have mostly a wide melting range, and only those which have high melting points will solidify to a sufficiently high degree at this temperature.

As described above, the samples were cooled at  $-70^{\circ}\text{C}$ . At this temperature the fats have a considerable tendency to separate in

unstable modifications. If the melting point is determined immediately after the cooling, a too low melting point will be found. This is due to the lower melting point of the unstable modifications. Perhaps also amorphous ("glassy") modifications can appear, these are not crystallised at all and "melt" below the melting point of the crystalline modifications.

Therefore the sample has to be stored for at least one day at  $0^{\circ}\text{C}$ . At this temperature a large part of the unstable modifications transform to the stable ones, and probably also the "glassy" solid turns into more stable crystalline forms. It is not necessary that all the fat is transformed into the stable modifications before the heating for melting point determination begins.

Further the polymorphous phenomena are less pronounced in fats of heterogeneous composition, *i. e.* containing widely different glycerides, such as human fats mostly do. They are extremely pronounced *e. g.* in cocoa butter which has a relatively very simple glyceride structure. Further the allotrope conversion takes place at a more rapid rate at higher temperatures. In the fat sample investigated in fig. 2 (melting point  $36.5^{\circ}\text{C}$ ) these conversions could not be followed by the dilatometric method above  $23^{\circ}\text{C}$ . If there were any, they had gone to an end before temperature equilibrium was reached in the dilatometer. Near the melting point the conversions go so rapidly that they are of no importance. It is only of importance that the major part of the fat is not present in the unstable condition when the heating begins, in this case a too low melting point cannot be avoided when heating at a tolerable rate.

### The Solidifying of Fats.

The thermodynamic equilibria are reached very slowly at low temperatures because of the slow conversion of the unstable modifications. At high temperatures the thermodynamically stable condition is reached rapidly when the temperature is rising. On cooling, on the contrary, the thermodynamic equilibrium will often be reached only after a very long time even when the temperature is high. This is due to the low tendency of the fats to separate out from mixtures. When a fat is cooled down to a temperature just below the melting point, it may keep fluid for a very long time.



Fig. 3. Crystals appearing in a melting point capillary kept moderately cooled during a period of several weeks.

The "solidifying point", the temperature at which the solidifying begins when the sample is cooled is always found to be lower than the melting point. The two constants represent the same thermodynamic equilibrium and ought to be the same if time is given for adjustment of the equilibria.

The "solidifying point" observed depends upon the time and rate of cooling, and if this constant shall be of use, the experimental conditions must be very well fixed and constant from one determination to the other. This "constant" gives a certain information concerning the tendency of the fat to tolerate undercooling without separating solids.

The human fats possess a considerable tendency not to solidify when moderately cooled below the melting point. A determination of the "solidifying point" will always differ several degrees from the melting point when it is determined according to the common methods, it can even differ ten degrees or more.

That the solidifying point is really identical with the melting point was demonstrated in the following way. Fat samples were placed in closed ampoules in thermostats, each of them at a temperature only some tenths of a degree (not more than  $0.5^{\circ}\text{C}$ ) below the melting point found for the sample in question. In all the cases crystals separated in the fat after some time. Among the 25 samples investigated in this way no one was found in which crystals did not separate out, but it required long time, in some samples more than a month was necessary.

The appearance of such a sample is shown in fig. 3. The crystals do not disappear when the sample is heated a little again. When the rise in temperature is only moderately above the melting point, very long time is needed for the redissolution. Probably

this solid fraction is the same as that which is present in the microcrystalline state in a sample just below the melting point when this constant is determined as described above.

When a sample to be used for melting point determination is only cooled to  $0^{\circ}\text{C}$ , relatively large crystals, similar to those here described, can separate. They will prevent the accurate determination of the melting point.

### Summary.

The "melting point" of fats generally means the upper limit of the temperature range through which the melting of the fat takes place, or, when determined according to empirical methods, a point very near this.

When the melting point of human fats were determined according to the common methods the results were not reproducible.

Therefore the melting process was investigated by following the changes during the melting process in some samples of human fat. It was found that the difficulties in the determinations on human fats were due to the characteristic that even several degrees below the real clear point only a very small fraction of solids is present. A fat as *e. g.* coconut oil has 3 per cent solids at  $0.3^{\circ}\text{C}$  below the upper limit of the melting range. In a human fat the solid fraction of 3 per cent was present at  $6^{\circ}\text{C}$  below the upper limit of the melting range. This means that the viscosity of this fat is low, even at a considerable distance below the "clear point". Therefore the empirical "constants" which are characterized by a certain low viscosity of the sample, *viz.* the softening point, the slipping point etc. are found far from the clear point and varying much with small variations in the external conditions.

When a sample of human fat to be used for melting point determination solidifies at  $0^{\circ}\text{C}$  heterogeneous crystals separate, which can produce large errors in the observed melting point. To avoid this the sample must be rapidly cooled down to an extremely low temperature ( $-70^{\circ}\text{C}$ ). In this way the fat solidifies as a microcrystalline mass which makes possible a relatively exact observation of the clear point. It was, by comparing with the dilatometric method, demonstrated that the clear point

determined according to the author's procedure is really identical with the thermodynamically fixed upper limit of the melting range.

The tendency of the fats to form unstable modifications with low melting points is discussed. Further it is demonstrated that from a thermodynamical point of view the melting point and the solidifying point are identical.

I wish to express my most sincere thanks to dr. HOFGAARD for his advice and assistance.

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## Melting Points of Human Fats as Related to their Location in the Body.

By

KNUT SCHMIDT-NIELSEN.

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During physiological investigations in relation to househeating problems (NIELSEN, 1946) it was found that the temperature of the extremities of man was often surprisingly low.

When the persons studied sat in a room of normal temperature ( $18^{\circ}\text{C}$ ) the temperature of the skin on the foot might very well sink to about  $20^{\circ}\text{C}$ — $25^{\circ}\text{C}$ . In spite of that the persons in question did not feel cold.

Previous investigations have shown definite relations between the characteristics of fats and the temperature at which the experimental animal lived (HENRIQUES and HANSEN, 1901). In this connection it would be of interest to know whether the low temperatures which often occur at the feet of human beings also induce low melting points in the subcutaneous fat.

The fat samples examined were taken from fresh material at The Institute of Forensic Medicine, The University of Copenhagen. The causes of death were traumatic or in some cases diseases of short duration.

Certain difficulties appeared in the melting point determinations. Most of the standard methods described in the literature gave results which were not reproducible.

The theoretical relations of the melting process of fats are discussed in another paper, in which also the method developed for melting point determinations in human fats is described (K. SCHMIDT-NIELSEN, 1946).



## Melting Points of Fat from Visceral Organs, Head, Arms and Legs.

Fat samples were taken from different places of the subcutis and visceral organs in five persons.

In the individual person the melting points of the fat from the different visceral organs were always about the same. The differences were only some few tenths of a degree. It is a common conception that the fat from the kidney capsula has an especially high melting point. As compared with fat from other visceral organs of the same person this was not the case, as compared with subcutaneous fat it was higher.

In the different persons the melting points from the viscera varied from  $32.6^{\circ}\text{C}$ — $36.6^{\circ}\text{C}$ . The subcutaneous fat from the front of the abdomen varied in the same five persons from  $31.7^{\circ}\text{C}$ — $34.9^{\circ}\text{C}$ .

About the same melting points were found in fats from the upper edge of the sternum, between the shoulder-blades, and at the arm at m. triceps. At the elbow the melting point was somewhat lower. It was impossible to obtain samples from the back of the hand, in samples from the inside of the hand the melting points  $19.8^{\circ}\text{C}$ — $26.8^{\circ}\text{C}$  were found. From the head samples were taken only at the temples just below the hair border. Here the melting points were from  $31.4^{\circ}\text{C}$ — $34.2^{\circ}\text{C}$ . This is considerably higher than in other peripheric parts.

In these five persons only few samples were taken from the legs. In three of the cases samples were taken from the inside as well as the outside of the ankle. The following melting points were found:

Ankle, inside	Ankle, outside
$13.5^{\circ}\text{C}$	$11.9^{\circ}\text{C}$
$25.9^{\circ}\text{C}$	$21.3^{\circ}\text{C}$
$13.9^{\circ}\text{C}$	$9.5^{\circ}\text{C}$

In these three cases the melting point is lower at the outside. This can possibly be put in relation to the fact that the large blood vessels to the foot pass down at the inside, while the outside is not well supplied with large vessels. To control if the temperature is generally higher at the inside, the temperature was measured at the ankles of several persons. The temperature of the skin was measured by means of thermo-elements, 2 cm below

the malleolus, simultaneously at the inside and the outside. This place corresponds to the place from where the fat samples were taken.

If the feet of the person in question were warm, there was no difference in the temperature of the skin at the two sides. If the feet were cold, the inside was always warmer than the outside. The difference was in many cases  $1-3^{\circ}\text{C}$ , but also larger differences were found. An exact determination of the differences which commonly occur require more systematic investigations.

### Melting Points of Fats from the Legs.

Samples were taken from the subcutis at different places at the lateral side of the legs from 15 persons. For comparison samples were taken from the omentum and from the abdominal subcutis in the same persons. The samples were designed by the following numbers:

- No. 1. Omentum.
- No. 2. Abdominal subcutis, near the umbilicus.
- No. 3. 15 cm below the iliac crest.
- No. 4. At the middle of femur.
- No. 5. Outside the knee-joint.
- No. 6. At the middle of the calf.
- No. 7. Below the lateral malleolus.
- No. 8. At the joint of the big toe.

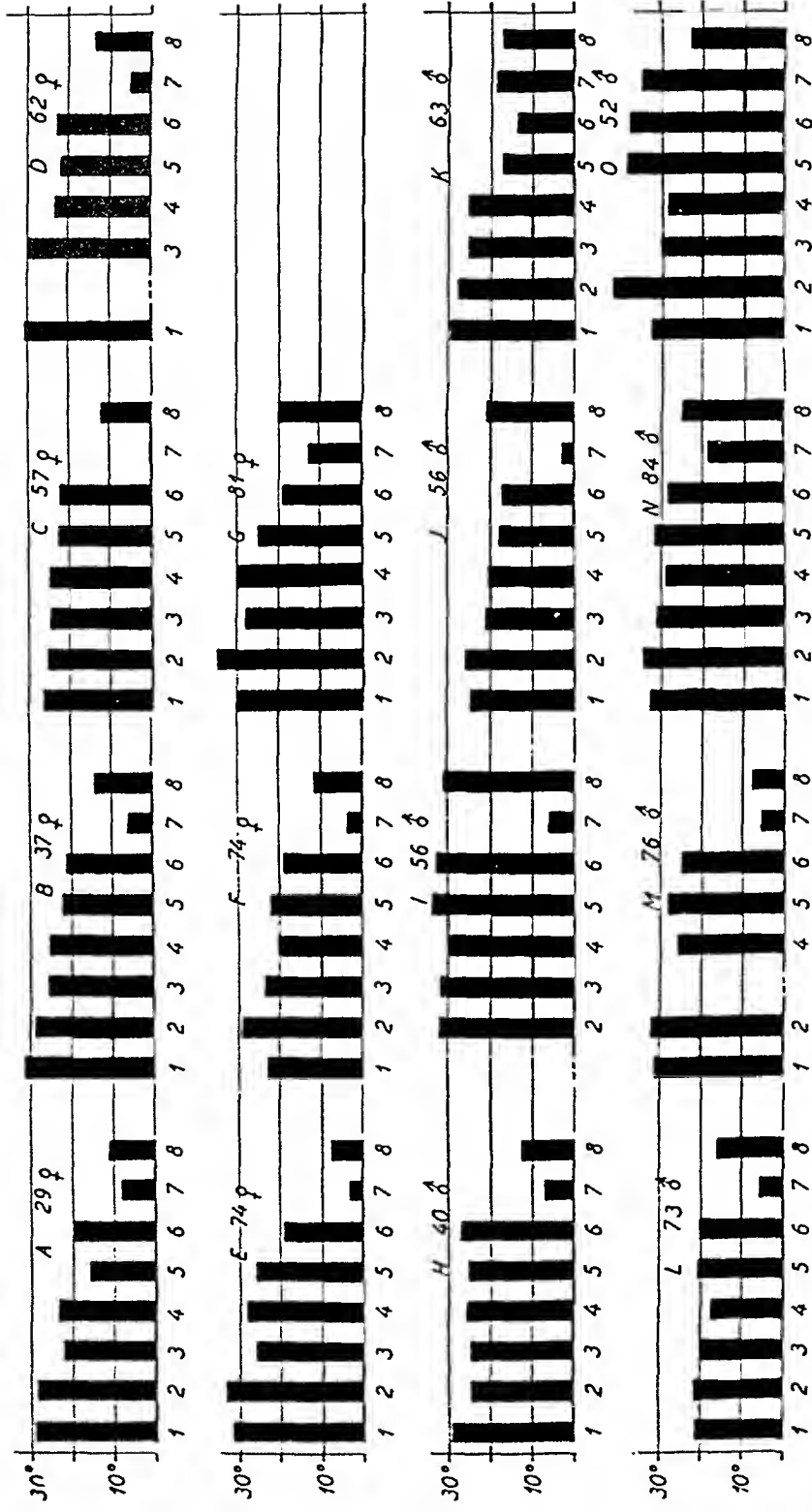
The total material investigated is given in the graph on page 126.

The most striking result is that the melting points of the fat samples are very varying. In the different persons as well as in the individual person the variations are very considerable. The highest melting point observed was  $41^{\circ}\text{C}$ , the lowest  $0.5^{\circ}\text{C}$ . In the individual person the variations may reach about  $30^{\circ}\text{C}$ .

*In all the persons lower melting points were generally found in the most peripheric parts.*

In the graph the men and the women are treated separately. The individual variations are too large for finding any difference according to sex or any other classification.

In all the persons (except the person O, who will be mentioned in detail below) the melting points in fat from the omentum and from the abdomen were about the same, about  $30^{\circ}\text{C}$ . The melting



Melting points in fat samples from 15 persons. The individual persons are designated by a letter, the age, and the sex. The melting points are given along the ordinate. The single samples are designated by numbers 1—8, namely: 1 = omentum; 2 = abdominal subcutis; 3 = 15 cm below the iliac crest; 4 = the middle of femur; 5 = outside the knee-joint; 6 = the middle of the calf; 7 = below the lateral malleolus; 8 = the joint of the big toe.

point may be lower at the abdomen than in the omentum. At the leg the melting points are mostly somewhat lower, and at the foot much lower melting points are often found. The lowest melting points are found at the lateral malleolus, the melting point at the big toe is generally somewhat higher. These low melting fats must be characterized as oils, they may even have so low melting points that they may be kept in the ice chest without solidifying or even without appearance of the slightest opacity (person C).

One of the persons gave results which were markedly different from those found in the other persons. This man (marked O in the graph) was a marine stoker, 52 years old. In this man all the melting points were extremely high. The highest value was  $41^{\circ}\text{C}$ , although in the other persons no value higher than  $34^{\circ}\text{C}$  was observed. The sample taken at the ankle gave  $34^{\circ}\text{C}$ , this place gives otherwise the lowest melting points. The sample from the big toe gave  $22.3^{\circ}\text{C}$ .

One cannot avoid to put these results in connection with the high temperatures at which this man had spent much of his time. As well known a marine stoker works at high temperature, and is in addition exposed to intense heat radiation. It would be very interesting to investigate further material of this character and decide if this exception from the general results really is caused by the extreme conditions during this man's daily work.

Another possibility to investigate if the temperature of the skin directly influences the melting point of the subcutaneous fat might perhaps be found in a certain group of neurasthenic persons. In many such patients one of the legs is always colder than the other, and the difference may be very considerable. If lower melting points are found at the cold leg, we can conclude that the melting point of human fats are influenced by the temperature of the tissue and not only by food or metabolic relations.

### Discussion.

HENRIQUES (1902) showed that the melting point (in fact the solidifying point was measured) in the subcutaneous fat from the pig is lower immediately under the skin, and that the melting point increases uniformly with increasing distance from the surface. Temperature measurements in the living pig also gave an increase from the surface inwards. HENRIQUES made an experimental verification of the influence of the temperature upon the

melting points. Fat from animals who had lived for two months at 30° C—35° C had a higher melting point (24.2° C) than that from animals at 0° C (melting point 22.8° C). Animals which lived at the low temperature, but were sewed in into sheepskin gave the highest melting point (25.2° C). The food had been the same in all animals, and the melting points in the fat from the viscera were the same in all of them.

S. SCHMIDT-NIELSEN and ESPELI (1941, a and b) investigated the marrow fat from ox, pig and reindeer. In the hind leg of the ox the melting point of the marrow fat were: 49.5° C in the femur, 40.4° C in the calf, and liquid in the foot. In the fore leg the melting point were similar, namely: 49.0° C, 40.0° C, liquid. The relations in the reindeer were similar to those in the ox. In the pig the relations were different. The melting points found in the same bones were 45.0° C—45.5° C—45.7° C in the hind leg and 45.1° C—45.5° C—45.6° C in the fore leg.

It is reasonable to put these results in connection with the temperature relations. In the pig the leg is short and the subcutaneous fat goes far down on the foot. One might expect that the temperature in the inner parts (the bones) does not sink much below the normal body temperature. The ox, on the contrary, has a relative long leg which is not protected by subcutaneous fat layers. One might expect that the temperatures in the periferic parts may easily sink considerably. The melting points found in the marrow fats in swine and in ox agree well with this assumption.

These findings, in connection with the demonstrated possibility of influencing the melting points of the fats by temperature changes (HENRIQUES), give much support to the supposition that the melting points in human subcutaneous fats are really influenced by the temperature.

### Summary.

Melting point in human fats varied between 41° C and 0.5° C. The largest variations in the individual person was about 30° C.

The melting point of visceral fat was 30° C—35° C. In the more periferic parts the melting points were lower. At the foot melting points of from 0° C—10° C were often found, in 11 out of 15 persons the melting point was below 10° C.

Measurements of the temperatures at men's feet show that low temperatures often occur. Since HENRIQUES experimentally demonstrated the connection between temperature and the melting point in subcutaneous fat, it can be supposed that the temperature at men's feet influence the melting point of the fat.

One single person was examined in whom the results were widely different from the others. He was a sea stoker, exposed to high temperatures in his daily work, and all the melting points observed in this man were extremely high.

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## The Effect of Hemorrhage and Shock on the Calibre of the Abdominal Vena Cava.

By

B. P. SILFVERSKIÖLD.

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In this series of investigations into the collapse problem the central vascular area (the heart; the aorta and vena cava with their largest branches) was studied with two methods: a modified Welcker method (SILFVERSKIÖLD 1938) and a roentgenological method (SILFVERSKIÖLD 1938, HEMMINGSSON and SILFVERSKIÖLD 1940, HOLMGREN and SILFVERSKIÖLD 1946). The area showed very considerable volume variations with the two procedures: both are, however, somewhat indirect.

The thoracic vessels are practically inaccessible to inspection, but the abdominal vessels can be examined directly and in the present work the reactions of the abdominal vena cava were investigated in this way.

*Method:* Rabbits were narcotized with urethane. A glass catheter was filled with heparin and inserted into the jugular vein. The abdomen was opened in the median line, and the intestines pushed aside, so that the vena cava could be studied. The catheter was now pushed down through the vena cava to the part which was to be studied, *i. e.* somewhat below the liver.

In its upper part the vena cava is a very big vessel. In rabbits weighing 2.5 kg the diameter of vena cava is about 8—9 mm between the points of entry of the kidney veins. Higher up it becomes wider, downwards it is much narrower. The calibre variations in a relatively wide portion of the cava were the most interesting from my point of view.

The width of the vena cava can best be measured when the vessel has been freed from the peritoneum, but it can also be studied with the vessel *in situ*. It is always preferable not to do much dissecting in the neighbourhood of the veins, as they are very sensitive to irritation and constrict very inconveniently. All preparations have to be made care-

fully, since rabbits are very exposed to shock from the abdominal operation and, as described lower down, the cava becomes narrower as a result of this kind of shock.

### Hemorrhage.

Two series of investigations were made. In the first of these 8 rabbits were subjected to a considerable hemorrhage through a cannula in the carotid artery. The arterial pressure fell after a relatively small loss of blood, and somewhat later the venous pressure began to fall as well.

The reduction of the cava pressure was never great, however; after a relatively severe hemorrhage, the decrease in pressure was about 2 cm H<sub>2</sub>O. (The rabbits were probably in a "subshock" condition before the bleeding, due to the abdominal operation, and therefore they could not stand any very great loss of blood.) Simultaneously with the fall in venous pressure the calibre of the vena cava decreased, and when the venous pressure had reached its lowest point, the vena cava had become markedly narrower: 4—5 mm in the part where its width had previously been 8—9 mm. This of course implies a very considerable reduction of the volume.

The reduction is not caused by simple collapse of the cava, as could be shown in two ways. The first was to produce a stasis by compression of the cava higher up than the part under observation (by pressure on the liver). By this method the pressure in the part of the vena cava studied could be raised some centimeters above the original level. In spite of this, only a very small dilatation of the cava followed.

The second way was to inject saline rapidly into a femoral vein. This did not dilate the vessel appreciably either, although the pressure was increased, as with the stasis method.

Thus the constricted vena cava could not be dilated by a moderate increase in its inner pressure (of short duration — about 30 sec.). A greater increase did of course enlarge the vessel, its wall being quite thin in the rabbit.

### Shock.

In this series of experiments a kind of shock was brought about by squeezing the intestines in 5 rabbits, prepared as in the foregoing series. The arterial pressure fell quite rapidly after this



squeezing, and the venous pressure slowly fell. The calibre of the vena cava diminished greatly, as in the hemorrhage experiments, and exactly as in those experiments, a moderate increase in the cava pressure did not bring about a restoration of the vessel volume, which was tested with stasis by liver pressure and with injections of saline.

Thus this kind of shock, too, produces an active constriction of the cava.

### Discussion.

FRANKLIN and Mc LACHLIN (1936) showed that faradic stimulation of the thoracic part of the sympathetic chain causes a constriction of the upper part of the abdominal cava and that a stimulation of the splanchnic nerve brings about a constriction of the lower part. The abdominal cava is also constricted by adrenaline.

The fact that a constriction of the abdominal vena cava can be provoked by electrical stimulation of its nerves suggested the probability that the diminution in calibre due to shock and hemorrhage is caused by a reflex.

Such a reflex might be released *via* the carotid sinus, as the mesenteric veins reacted to reflexes from the carotid sinus in the investigations of HEYMANS, BOUCKAERT and DAUTREBANDE (1930), FLEISCH (1930), GOLWITZER-MEIER and SCHULTE (1931). Clamping of the carotid arteries, however, did not cause any definite constriction of the cava; in any case the constriction was not of the same order as in hemorrhage and shock.

Therefore it is possible that some other mechanism is responsible for these vessel reactions — perhaps some complicated, direct effect of the low venous pressure on the vein wall.

Another proof of the active nature of the calibre reduction was the fact that tapping of the cava with the forefinger as described by FRANKLIN and Mc LACHLIN (1936) produced a prolonged dilatation to its original size (without any increase in the caval pressure).

### Summary.

A severe loss of blood produces a great reduction in the width of the abdominal vena cava.

Likewise shock caused by squeezing the intestines is followed by a great diminution in the calibre of the abdominal cava.

In both cases the width reduction was produced by an active constriction.

The constriction was evidently not caused by a carotid sinus reflex.

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## The Volume Variations of the Large Central Blood Vessels.

By

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Earlier investigations (HEMMINGSSON and SILFVERSKIÖLD 1940; SILFVERSKIÖLD 1938) showed that the large thoracic vessels (and the adjacent parts of the vessels of the neck and the anterior extremities) together with the heart contain a large proportion of the total blood of the body (about 30 % in mice, 26 % in rabbits). If the abdominal parts of the vena cava and aorta are added, the volume of the central vessels becomes still more striking.

It was evident that in the animals examined the volume of these central vessels was approximately as large as that of all the abdominal viscera together and the volume variations were great.

The new data afforded by these experiments lie in the quantitative results. That the heart and the adjacent vessels may decrease in size is known from clinical experience (orthostatic reactions, hemorrhage). Further information as to the reactions of the big vessels has been gained by experimental work (see FRANKLIN 1937; SCHRETZENMAYER 1936; GIANTURCO and STEGGERDA 1937).

This investigation was carried out in order to acquire more detailed knowledge of the pathophysiology of the vessel reactions. For that purpose we have decided to continue the study of the volume variations due to loss of blood and those following on an acute intoxication (ether).

## Hemorrhage.

As mentioned above, the central vessels decrease in volume after loss of blood. GIANTURCO and STEGGERDA (1937) state briefly that the big vessels are emptied by hemorrhage; the abdominal vessels were emptied before the rest of the circulatory system. SILFVERSKIÖLD (1938) found that after a heavy hemorrhage, the quantitatively most important compensatory reduction in size takes place in the big vessels. Even if this were merely a passive adaptation to a sinking inner pressure it would be of importance for the compensation of the blood loss. It would be more interesting, however, if the width reduction were of an active nature.

*Method.* Experiments were carried out on 11 rabbits. They were anesthetized with 20 % urethane injected intravenously (7 cc/kg). Blood clotting was prevented with heparine. A cannula was inserted into the carotid artery, and a catheter pushed through the jugular vein to vena cava superior or inferior. A special cannula was inserted into the pleural cavity, hermetic closure being secured by sewing the thoracic muscles round it.

The vessel reactions were studied after visualization with thorotrast (thorium dioxide). 12 cc thorotrast were injected intravenously, which did not appear to hurt the animals. Before the injection roentgen photos were taken, and in several instances the big vessels were seen clearly enough to be compared with the contrast pictures (which were taken some minutes after the injection, when the venous pressure had returned to the normal level). In these instances it was found that the vessels were not dilated after the injection of thorotrast, which was considered to be a fact of great importance.

The animals were bled through the artery cannula twice or more. At first about  $1/5$  of the total blood volume was taken, the second time somewhat less. As a rule the animals seemed to be moderately affected by the hemorrhage, and they could be restored by reinjection of the blood.

*Result:* Fig. 1 shows how strong the diminution in calibre may be. The aorta and the heart decrease in the first place and to the greatest extent; the vena cava sometimes only after the second hemorrhage. — Most of the pictures were taken with the animal in the lateral position, which afforded the best visualization of the big vessels. Some roentgenograms, however, were taken with the animals rotated 45 degrees, in order to show that the width reduction was concentric (front pictures were of little value as the vessels were covered by the heart).

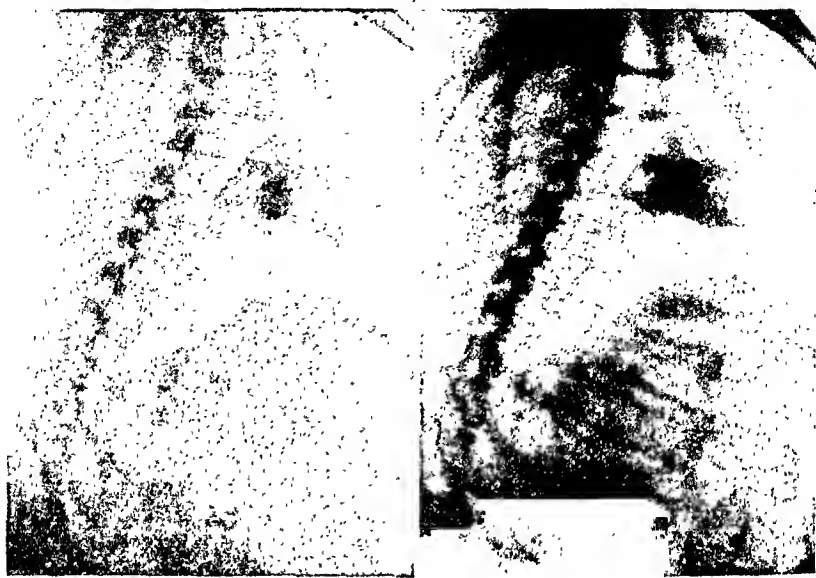


Fig. 1.

- a. The big central vessels (of a rabbit) after permanent visualization with a relatively small amount of thorium dioxide.
- b. After a hemorrhage ( $1/5$  of the total blood volume) the heart, the aorta and the abdominal part of the vena cava have decreased in size.

The outlines of the vessels were still sharp after the hemorrhage, and obviously the picture of reduction in size was not the result of a dilution — by inflow of tissue fluid into the blood. (See also controls by SILFVERSKIÖLD 1946.)

As seen in the figures, the decrease in volume also comprised the abdominal parts of the vena cava and aorta; the vena portae diminished in width in some of the experiments, in others not.

It was evident that the outer and inner pressure (the "pleural" and venous pressure) of the thoracic part of the inferior vena cava changed very little. Therefore the reduction in calibre of the vena cava is not likely to have been the result of a simple collapse of the vessel. The thoracic part of vena cava is provided with very few muscle fibres (FRANKLIN 1933) and therefore the constriction in this part may be very weak. The reactions of the abdominal vena cava have been studied in detail by SILFVERSKIÖLD (1946).

The inner pressures of the heart (at least that of the ventricles) and of the aorta sink of course during the hemorrhage and there-

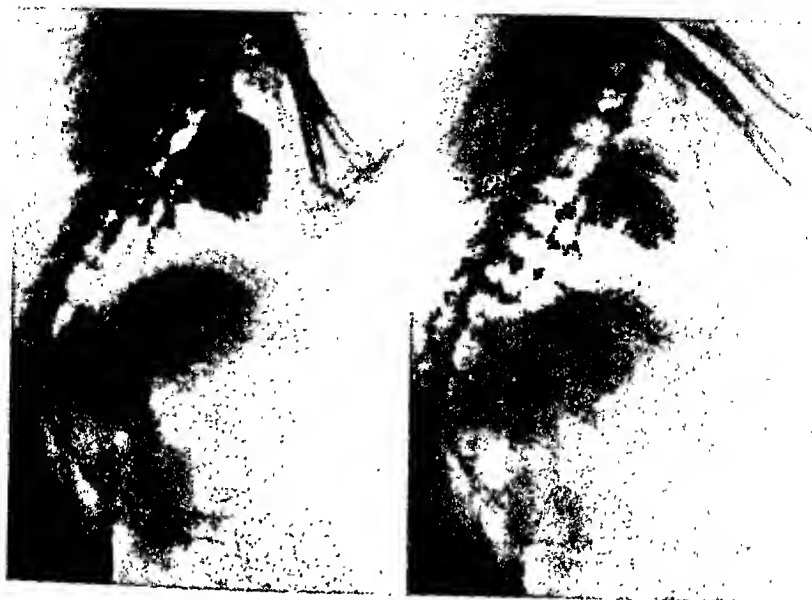


Fig. 2.

- a. The big central vessels.  
 b. Ether intoxication: the picture was taken immediately after stop of the respiration. The aorta has diminished in size; the vena cava has become somewhat wider, without any increase of the venous pressure.

fore it cannot be decided whether their diminution in size is to some degree the result of an active procedure.

Roentgenograms from the vessels of the caudal part of the body before and after hemorrhage showed that the big skin vein exhibited in some cases a small decrease in width, in other cases there was no decrease at all.

We have not made a detailed study of the reactions of the various vascular areas of the body, but from the roentgenograms of the caudal part it was evident that the skin vein reaction differed from that of the vena cava.

It can be added that the vessel contraction after severe hemorrhage persists a long time and therefore it is not a question of a temporary volume reduction of the big vessels.

### Ether Intoxication.

The blood distribution in cases of severe ether intoxication seems to be the same as in many other acute intoxications. The

liver and the kidney become strongly hyperemic, while the blood of the spleen and also of the mesenteric vessels decreases. This could be verified by quantitative measurements on mice (SILFVERSKIÖLD 1938). In the same work it was pointed out that the great increase in the blood volume of the liver was not compensated by contraction of the central vascular area, but blood was drawn from the head and the legs; similar result was seen in various acute intoxications.

GIANTURCO and STEGGERDA (1937) studied with X-rays the width of the big vessels after various experiments. In most instances they found that the aorta became narrow, while the cava was wide and the liver congested. They thought that this might be ascribed to aorta constriction (efedrine), impaired heart function (air embolism), constriction of the pulmonary vessels (histamine), and so on, and that the vena cava had been dilated by stasis. However, they purposely used very heavy or lethal doses of the drugs, and therefore the vascular picture may have been that of the severe acute intoxications mentioned above. The narrowing of the aorta may have been caused by collapse and not of an active constriction. Without pressure measurements it cannot be decided whether a volume change in a vessel is an active or a passive one.

In the present investigation we measured the pressure of the vena cava during ether intoxication, in order to find out whether the absence of decrease in volume (HEMMINGSSON and SILFVERSKIÖLD 1940) was the result of stasis.

*Method.* The rabbits were narcotized with urethane and a catheter inserted into the vena cava, as described above. Pictures were taken before and after the thorotrast injection, and in some cases also soon after the beginning of the administration of ether. After that the animals were deeply narcotized with ether, and fresh pictures were taken after the cessation of respiration. In this stage the heart continued beating. — 9 rabbits were examined.

*Results:* With this ether intoxication the vena cava and the heart do not decrease in width (as seen in figure 2). Sometimes they are enlarged a little. The aorta, however, becomes very narrow. The pressure of the vena cava does not increase at this stage (but somewhat later there is an increase), and thus there is no stasis, a fact of considerable importance.

From the roentgenograms of the legs it was seen that the big skin vein had constricted to such a degree that it could hardly

be seen. Other peripheral veins also seemed to be constricted. From this the conclusion can be drawn that there may be a great difference in reaction between the peripheral veins and the central ones.

### Discussion.

The width of the vena cava may vary independently of that of the peripheral veins, and the volume variations of the cava are not simply the result of collapse or stasis. Whether the variations in the size of the heart and the aorta are to be explained as being of an entirely passive nature cannot yet be decided. The volume of the heart is, however, influenced by reflexes from the carotid sinus (HEYMANS and BOUCKAERT 1930) and therefore it seems reasonable to believe that the volume reduction in our experiments was partly caused by a reflex action.

In certain conditions, however, the vessels mentioned may have a blood depot function (4, 8). In normal individuals in the upright position the heart contains a smaller volume of blood than in the recumbent position — an old experience from clinical roentgen examinations. NYLIN (1934) found an average reduction of 250 cc, which is a high value, and probably too high. The big veins of the chest and their branches certainly also become narrower as a result of the reduced hydrostatic pressure (probably other vessels of the upper part of the body get narrower, too). Thus there must be a considerable quantity of blood in the central vessels of the recumbent individual which is not absolutely needed for the circulation.

The "superfluous" blood may be involved in hemorrhage, shock and perhaps also in other conditions. This of course does not imply that the central vascular area has all the various depot properties of the spleen.

### Summary.

The volume variations of the big central vessels (vena cava and aorta) and the heart were studied in animal experiments employing a roentgen method.

This central vascular area showed considerable width variations after severe hemorrhage and ether intoxication. The vena cava reactions could not be explained as being of an entirely passive nature.

There are reasons for ascribing a real blood depot function to the heart and the big vessels.



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## The Salicylaldehyde-method as a Micromethod for the Determination of Total Acetone in Capillary Blood.

By

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The total acetone content of the blood is made up by two fractions: The acetone performed and the acetone formed by quantitative decarboxylation of the acetylacetic acid. For its quantitative determination the acetylacetic acid is first decarboxylated, and then the total amount of acetone is determined. The determination of acetone may be carried out according to several principles. When operating with capillary blood, however, not only accuracy and specificity, but even sensitivity is required from the method employed. The principal methods for the determination of acetone are indicated below.

1. The iodometric method of MESSINGER (1888) and modifications. The acetone is distilled off into a flask containing an alkaline iodine solution. Hereby it is converted into iodoform. After acidifying the solution excess of iodine is determined by titration. But as even many other volatile substances are consuming iodine, the specificity of the method is low. If demanding any accuracy, amounts of acetone greater than 10  $\gamma$  in the sample are required. For the determination of total acetone in small blood samples this method is of no use.

2. Methods based on the precipitation of acetone with mercury salts. DENIGÈS (1898) found that when boiling acetone with a solution of mercuric sulphate in sulfuric acid, a crystalline precipitate separated. He used this reaction for the determination of acetone. Later this method is modified by VAN SLYKE and collaborators (VAN SLYKE 1917; VAN SLYKE and FITZ 1917 and 1919). BARNES and collaborators (BARNES 1937; BARNES and WICK 1939) have combined this precipitation

with a distillation afterwards and determination of acetone in the fluid distilled off by means of the iodometric method. The determination of acetone by precipitation with mercuric sulfate is more specific than the iodometric method of MESSINGER, but considerably less sensitive. In order to obtain satisfactory results, quantities of more than 50  $\gamma$  acetone must be present in the sample. The method is therefore not applicable when operating with very small blood samples containing only a few  $\gamma$  of acetone or even less.

Acetone can also be precipitated with mercuric cyanide in alkaline solution. This is the base of a quantitative method, introduced by SCOTT-WILSON (1911). Nephelometric modifications are introduced by MARRIOTT (1913—14), FOLIN and DENIS (1914), and SHIPLEY and LONG (1938). The nephelometric modifications are rather sensitive, but errors are easily magnified — up to several hundred per cent (BARNES and WICK 1939) — like in most nephelometric measurements. The method is therefore of no use when accurate results are wanted.

3. Methods based on the precipitation of acetone with the reagent of NESSLER. Even by this reaction the acetone is nephelometrically determined (ABELS 1937; WERCH 1940 and 1941). The sensitivity of this method is large, but errors are large too, particularly when the analyses are carried out by unexperienced investigators.

4. Colorimetric methods based on colour reactions between acetone, an aromatic aldehyde, and alkali. Various aldehydes are used. When using benzaldehyde, dibenzalacetone is formed (CLAISEN 1884), but this reaction is hardly suitable for the quantitative determination of acetone. The corresponding reaction with furfural, however, is employed for the determination of acetone, lately by CASTIGLIONI (1941). The reaction with o-nitrobenzaldehyde (PENTZOLD 1883) has been used by KIESEL (1905) and lately by NOYES (1941). Also with vanilline a dye is formed, and also this reaction is employed for acetone determination (BÄCKSTRÖM 1942). All these reactions are relatively specific, but the presence of acetaldehyde and some other substances will disturb the determination. Methods based on the reactions mentioned above are further considered not to be sufficiently sensitive for analysis of capillary blood samples, where the acetone occurs in very small quantities.

When treating acetone solutions with salicylaldehyde and alkali, a dye is also formed (FABINYI 1900). This reaction was used by FROMMER (1905) as a very sensitive indicator of the presence of acetone in urine. A rough quantitative application of this reaction is described by ENGFELDT (1915). He indicates that even with concentrations of about 2  $\gamma$  acetone in 10 ml solution a visible colour is obtained. The salicylaldehyde method for the quantitative determination of acetone has later been improved by a number of investigators (CSONKA 1916; BEHRE and BENEDICT 1920; URBACH 1931; NEUWEILER 1933, and many others).

For the determination of acetone the salicylaldehyde method seems to be the most specific and most sensitive of all the methods available. But in the literature available at present we have found no modification of this method suitable for the analysis of small blood samples, *e. g.* samples of capillary blood. For this reason we have found it justifiable to publish a micromodification of the salicylaldehyde method suitable for exact determination of total acetone in samples of capillary blood.

The analysis is based on the following principle: The blood is collected in diluted sulfuric acid and the mixture afterwards distilled. Hereby the acetylacetic acid is quantitatively decarboxylated, and the total acetone content of the sample is subjected to distillation. Potassium hydroxyde and salicylaldehyde solutions are added to the distilled fluid. A control test is prepared in the same manner by using distilled water instead of the acetone-containing fluid. In order to develop the colour the flasks are heated on a water bath. Finally the extinction is determined in a Pulfrich Photometer.

We have found that no precipitation of proteins is necessitated before distilling off the total acetone. Samples in which the proteins had been removed according to FUJITA and IWATAKE (1931) and samples not treated in this manner revealed essentially the same results. — As to the concentration of the sulfuric acid employed, this is of little importance. Any concentration of the acid between  $n/25$  and  $1\ n$  will give identical results. — The distillation should preferably be undertaken in especially designed apparatus, but may be carried out in any equipment designed for similar analysis. The equipment used by the authors is reproduced in the fig. 1.

The tube filled with glass beads has a dual purpose:

1. It will retain foam inevitably formed by the boiling fluid.
2. It will facilitate the fractionation of the acetone. Thus a small quantity of fluid distilled off will contain all the acetone present in the sample.

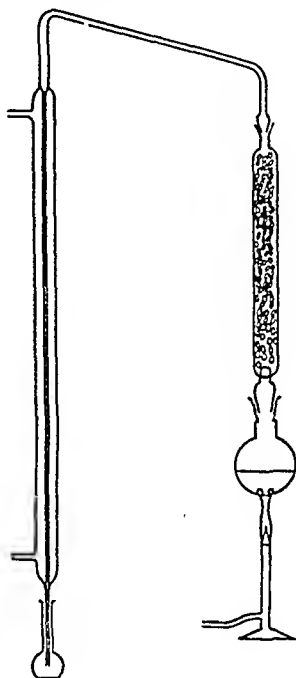


Fig. 1.

### Reagents.

1. Dilute sulphuric acid, approximately N/5.
2. KOH, exactly 14.5 N/solution.
3. Salicylaldehyde solution. To 10 g salicylaldehyde alcohol is added to 100 ml.

Note. To avoid errors due to acetone in the distilled water this must be boiled for half an hour before use.

### Procedure.

If maximum of accuracy is required, the blood sample must contain at least 0.5  $\gamma$  acetone. For normal individuals a blood quantity of 0.2 ml will give satisfactory results. During keton-aemia 0.1 or 0.25 ml will be sufficient.

The blood sample is collected in a 100 ml flask fitted with a glass-ground joint, containing 25 ml dilute sulphuric acid. To prevent bumping a few glass beads are added. Then the flask is connected to the distillation equipment, and the flask is heated by a gas burner. The acetone distilling off is collected in a 10 ml volumetric flask. We have found that in 10—15 minutes all the acetone is distilled off. During this time heating is regulated in order to obtain a quantity of approximately 4 ml of fluid distilled. Attention should be paid to the cooling arrangement. No drops should be permitted to remain inside the cooler. A blind is prepared by distilling 25 ml of the sulphuric acid employed.

After distilling all the samples the acetone content in the volumetric flasks is determined. To each flask (blind included) 4 ml KOH and 2 ml salicylaldehyde solution are added. The mixture is made up to volume with water. The flasks are stoppered and heated on a water bath at 50° for 20 minutes. Afterwards they are allowed to cool at room temperature for half an hour. The extinction is finally determined in a Pulfrich photometer in 50 mm "Klein-Küvetten", fitted with covers. Filter S 53 is used. In order to obtain exact values the extinction should be measured within an hour after cooling the flasks. Under these circumstances exact proportionality will exist between the acetone content of the volumetric flask and the extinction (Krog 1945). If the cuvettes are left standing for some time before being read in the photometer, they should be inverted several times;

the alcohol evaporates rapidly, condensing on the inner surface of the cover. Errors may thereby result.

**Calculation.** To some extent the acetone concentration corresponding to an extinction  $E = 1.00$  depends upon the salicylaldehyde employed. It has to be determined by means of an acetone solution of known concentration. Using a salicylaldehyde preparation produced according to GATTERMANN (1930), we found that  $E = 1.00$  was given by an acetone content in the volumetric flask of 6.7  $\gamma$ . Thus the acetone contained in the sample will be found by multiplying this factor with the extinction of the sample. On the basis of the quantity of blood employed the total acetone per ml may be calculated.

Some values found by the method are given below. The samples nr. 7 and 9 originated from persons suffering from inanition ketonaemia, the others from healthy persons. Each analysis is carried out with 0.2 ml of blood.

Sample no.	Total acetone of the sample, $\gamma$		$\gamma$ acetone added to 0.2 ml of the same blood	Total acetone after the addition, $\gamma$		Acetone recovered, %
	Paral- lels	Mean		Paral- lels	Mean	
1	0.81 0.86	0.84	3	3.79 3.82	3.81	99
2	0.67 0.69	0.68	3	3.58 3.62	3.60	97
3	0.89 0.92	0.91	3	4.03 3.99	4.01	103
4	0.78 0.84	0.81	3	3.72 3.70	3.71	97
5	1.20 1.15	1.18	2	3.17 3.18	3.18	100
6	0.73 0.76	0.75	2	2.73 2.71	2.72	98
7	1.75 1.81	1.78	2	3.69 3.75	3.72	97
8	0.53 0.61	0.57	2	2.54 2.56	2.55	99
9	2.42 2.44	2.43	2	4.41 4.44	4.43	100
10	0.77 0.80 0.76	0.78	2	2.82	2.82	102
	"	"	1	1.81	1.81	103

### Summary.

A micromodification of the salicylaldehyde method for total acetone determination in small blood samples is described. Total acetone quantities of about 1  $\gamma$  in the sample may be determined with great accuracy.

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## Über Gültigkeit und Grenzen der bilinearen Reizausdrücke bei schwellenmässigen Lichtempfindungen.

Von

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Die Frage von der gegenseitigen Bedeutung der begrifflichen Bestimmungsstücke eines Reizausdrucks an Hand des Reizausdrucks kurzdauernder Lichtempfindungen hat einer von uns in dem Buch »Über Wahrnehmen, Denken und messendes Versuchen« zu erörtern versucht. In der vorliegenden Arbeit sollen diese Versuche mehr detailliert beschrieben werden, desgleichen neue, während des Krieges ausgeführte Versuche über die Bestimmungsstücke von Lichtreizen von verschiedener Flächengrösse, insbesondere von ganz kleiner Fläche.

Die eigenartige Stellung der begrifflichen Zeitgrösse geht aus allen, in den verschiedensten Modalgebieten ausgeführten Versuchen hervor, in denen sich die Zeit unter den gemessenen Reizbestimmungsstücken befindet. Auf dem Gebiete des Gesichts haben bekanntlich BLOCH (1885), CHARPENTIER (1890), v. KRIES (1907) und PIÉRON (1932) gezeigt, dass in den Reizausdrücken, die der absoluten Lichtschwelle entsprechen, das Produkt der Grössen Lichtintensität (welche eine Grösse von der Dimension einer Leistung ist) und Zeitdauer konstant ist. Wenn die Lichtintensität oder -Leistung mit  $L$  und die Zeitdauer mit  $t$  bezeichnet werden, gilt also hier:  $L \cdot t = E_1$ , wobei  $E_1$  die konstante Energie des Minimalreizes der absoluten Schwellenempfindung bedeutet. Diese Regel gilt genau bis zu Zeitdauern des Reizes, die höchstens etwa 100 bis 120  $\sigma$  betragen. Bei den Versuchen war die



konstant gehaltene Reizfläche von einer solchen Grösse, dass ihr Bild ganz innerhalb der Fovea centralis fiel.

Auch auf den Gebieten des Gehörs und der Hautempfindung (beim elektrischen Hautreizen) gelten ähnliche Regeln beim Beobachten der absoluten Empfindungsschwelle der betreffenden Gebiete. Auch hierbei kompensieren die Leistungs- und die Zeitgrösse einander in der durch die Produktenregel angegebenen Weise, wie von verschiedenen Autoren festgestellt worden ist (NERNST 1908; KUCHARSKI 1928; WÄRE, WILSKA u. RENQVIST 1933).

Die obigen Produktenregeln geben eine bestimmte Eigenheit des Observierens von Minimalreizen (es handelt sich in allen den genannten Fällen um absolute Empfindungsschwellen) an. Die Gültigkeit der Produktenregel bedeutet nämlich, dass in diesen Fällen die Bestimmung des Zeitwerts bzw. des Leistungs- oder Intensitätswerts in dem betreffenden Modalgebiet unmöglich ist, d. h. die Beobachtung gilt in diesem Gebiet nicht der Intensität und der Zeitdauer, sondern der *Energie*. Man könnte hier von einer Entartung der Dimensionen innerhalb des betreffenden Modalgebietes sprechen. Statt der zwei Dimensionen, der Intensität und der Zeit, ist nur eine, und zwar die *Energie beobachtbar*. Die Einführung der Intensitäts- und Zeitgrössen in den Reizausdruck der Produktenregel geschieht auf Grund von Beobachtungen und Messungen, die nicht zum betreffenden Versuch gehören. Wir messen bei der Versuchsausführung, mittels besonderer technischer Hilfsmittel die Grössen Intensität und Zeit und konstatieren dann die Konstanz des Produktes dieser Grössen. Wenn wir statt dessen die physikalische Grösse des Schwärzungsgrades einer photographischen Platte entsprechend den absoluten Lichtempfindungsschwellen bestimmt hätten, würden wir die Regel: Schwärzungsgrad, d. h. Lichtenergie = konstant, erhalten haben. Die Energie als Reizgrösse genügt also, um in der einfachstmöglichen Weise begrifflich das phänomenale Ergebnis des Versuches zu wiedergeben; in dieser Beziehung liegt diese begriffliche Grösse bei diesen Versuchen der Phänomenalität am nächsten. Die in den Produktenregeln stehenden Grössen sind der Phänomenalität der Minimaempfindung fremd und die Entdeckung, dass ihr Produkt bei der Minimaempfindung eine Konstante ist, erscheint einem als ein Wunder. Wir haben die Produktenregel eine sinnesphysiologische *Einschränkungsrelation* und dasjenige Gebiet, wo die Intensität und die Zeit nicht mehr getrennte Rollen spielen, das sinnesphysiologische *Einschränkungsgebiet* genannt.

Man kann dieser Einschränkung oder der Entartung der Dimensionen auch einen anderen Ausdruck geben. Wenn es sich um eine *bestimmte Empfindungsschwelle* handelt, z. B. um die absolute Empfindungsschwelle, wie in den vorigen Fällen, so besteht im Einschränkungsbereich zwischen zwei (wie in den vorigen Fällen) oder zwischen mehreren Reizgrössendimensionen eine Gleichung, durch welche z. B. eine von den Dimensionen bestimmt werden kann, wenn die übrigen gegeben sind. Die Gleichung braucht natürlich im allgemeinen nicht die Form  $L \cdot t = E_1$  zu haben. Mit dieser Formulierung kann zugleich der Untersuchung ein erstes Ziel gegeben werden: die Aufstellung der betreffenden Gleichung. An diese Aufgabe schliesst sich als Nebenproblem die Bestimmung der Grenzen an, innerhalb deren die Gleichung gültig ist.

Eine der obigen Einschränkungsrelation ähnliche Beziehung gilt auf dem Gebiete des Gesichts auch betreffs der Grössen Lichtintensität und Flächengrösse der Belichtung. Für Flächen, die kleineren Knotenpunkts winkeln als etwa  $2'$  entsprechen, hat ASHER (1897) gefunden, dass die Beziehung  $L \cdot f = L_1$  gilt, wo die Intensität mit  $L$ , die Flächengrösse mit  $f$  und mit  $L_1$  eine konstante Grösse bezeichnet wird, die von der physikalischen Dimension einer Leistung ist. Bei den Versuchen von ASHER handelte es sich um »Dauerreize«. Eine Beziehung von dieser Form wird oft als RICCO'sche Regel bezeichnet. Wie gesagt gilt diese Regel nicht mehr bei grösseren Gesichtswinkeln.

Bei Dunkeladaptation gilt, wie PIPER (1903) gezeigt hat, bei der absoluten Schwelle die Regel  $L \cdot \sqrt{f} = \text{Konstante}$ , ziemlich genau, was später von anderen bestätigt worden ist. Kürzlich haben WALD (1937—38) und CROZIER (CROZIER und HOLWAY 1939—1941) genauer gezeigt, unter welchen Umständen gültig und von welcher Form die Ausdrücke sind, welche die Einschränkung der Reizbestimmungsstücke der Flächengrösse und der Intensitätsgrösse angeben. Bei ganz kleinen Gesichtswinkeln gilt annäherungsweise die Formel  $L \cdot f = \text{Konstante}$ , bei grösseren Flächen und bei der Bestimmung von Unterschiedsschwellen Ausdrücke von komplizierterer Form. Die an ihre Messungen sich anschliessenden Ausführungen von CROZIER und von WALD sind aufschlussreich und beleuchten von einem eigenen Standpunkt die besprochenen Verhältnisse.

Aus den beiden, bei der absoluten Schwelle gültigen Regeln  $L \cdot t = E_1$  und  $L \cdot f = L_1$ , also den Zeit- und Flächenregeln, folgt die verallgemeinerte Einschränkungsbereichsrelation  $L \cdot f \cdot t = E_1$  (= Kon-

stante). Die empirische Gültigkeit und besonders *die Grenzen* dieser Regeln werden in dieser Arbeit erneut einer Untersuchung unterworfen.

## Eigene Versuche.

### Untersuchung der Abhängigkeit der Reizgrössen Lichtintensität und Zeit.

Wir untersuchten zuerst die gegenseitigen Verhältnisse dieser Reizgrössen bei der absoluten Empfindungsschwelle mit Reizflächen, deren Bilder ganz innerhalb der Fovca centralis fallen. Dies war also eine Wiederholung der früheren von BLOCH bis v. KRIES und PIÉRON sich erstreckenden Versuche. Dagegen liegen bisher keine solche Untersuchungen vor, bei denen die Reizgrössen Intensität und Zeit untersucht worden wären, wenn es sich um *überschwellige* Empfindungen handelt. Solche Untersuchungen anzustellen war daher unser zweites Unternehmen. Und schliesslich wurden noch Bestimmungen über die Abhängigkeit der Reizgrössen Intensität und Fläche an der absoluten Empfindungsschwelle sowie an drei überschwelligten Stellen angestellt, also Versuche, die parallel mit denjenigen von ASHER, CROZIER, WALD u. a. gingen.

Die *Versuchsanordnung* ist schematisch in Abb. 1 angegeben. Mit Hilfe zweier Zeiss'scher Projektionslampen (P) sowie zweier Linsensysteme (L) mit Irisblenden (I) werden auf einen 3 m von der Versuchsperson entfernten weissen Schirm (S) zwei (oder nur eine) kreisrunde Lichtflächen projiziert. Die Feinregulierung der *Intensität* der Lichtflächen wird durch die Blenden ( $I_1$ ) abgestuft; die Grundeinstellung dagegen durch Vorschalten von Rauchgläsern (R). (Bei sehr genauen Unterschiedsschwellenbestimmungen wird die Intensität mittels sehr spitzer Rauchkeilgläser bewerkstelligt (K).) Da die Lampen vom Stadtnetz gespeist werden, wird die Stromstärke kontinuierlich mit einem genauen Milliampereometer kontrolliert und mittels eines vorgeschalteten, regulierbaren Widerstandes genau konstant gehalten.

Die *Grösse der Lichtflächen* wird mittels der Blenden ( $I_2$ ) eingestellt. Die von der Firma Zeiss vorzüglich ausgeführte Anordnung gestattet, Lichtprojektionen von völlig gleichmässiger Helligkeit zu erzielen, bis zu Flächengrössen von etwa 40 mm Durchmesser (in 3 m Entfernung). Mittels einer Hebelanordnung, welche die optische Bank (A), auf der eine der Projektionsanordnungen aufmontiert ist, um eine Axe dreht, kann der gegenseitige Abstand der Lichtflächen vergrössert und verkleinert werden. Die Flächen lassen sich hierbei auf einander schieben und andererseits in eine Entfernung von mehr als 70 mm von einander bringen.



Tabelle 1.

Gegenseitige Abhängigkeit der Reizzeitdauer  $t$  (in Sigmen  $\sigma$ ) und der Reizlichtintensität  $L$  (in Lux) an der *absoluten Empfindungsschwelle* der Stelle 1, welche gleich der Schwelle mit der Ordnungszahl 1 ist sowie an der Stelle 4, welche gleich der *Schwelle mit der Ordnungszahl 2,8* ist. (Siehe den Text.) Die Stelle 4 entspricht einer Empfindungsstärke, die *gleich* derjenigen ist, welche von einer Lichtintensität von  $0.096 L_{1x}$  mit der Zeitdauer von  $110 \sigma$  bewirkt wird. Diese Reize entsprechen der Intensitätsstellenordnungszahl 4 bei Dauerbelichtung. (Knotenpunktswinkel der Lichtkreise  $34'$ )

S t e l l e 1. (absolute, 1. Schwelle)			S t e l l e 4. (2,8 Schwelle)	
$t$ $\sigma$	$L$ $L_{1x}$	$L \cdot t = E_1$ $L_{1x} \cdot \sigma$	$L$ $L_{4x}$	$L \cdot t = E_4$ $L_{4x} \cdot \sigma$
1	3.40 $\pm$ 0.32	2.40	5.63 $\pm$ 0.60	5.63
2	— —	—	2.75 $\pm$ 0.30	5.50
4	0.454 $\pm$ 0.050	1.82	1.41 $\pm$ 0.31	5.64
10	0.249 $\pm$ 0.015	2.49	0.650 $\pm$ 0.085	6.50
15	0.179 $\pm$ 0.014	2.69	0.452 $\pm$ 0.037	6.78
20	0.162 $\pm$ 0.011	3.24	0.353 $\pm$ 0.032	7.06
40	0.0798 $\pm$ 0.0114	3.19	0.199 $\pm$ 0.027	7.96
60	— —	—	0.141 $\pm$ 0.015	8.46
80	0.0498 $\pm$ 0.0057	3.98	0.130 $\pm$ 0.0087	10.40
100	0.0270 $\pm$ 0.0046	2.70	0.113 $\pm$ 0.019	11.3
110	— —	—	0.119 $\pm$ 0.022	13.1
120	0.0285 $\pm$ 0.0049	3.42	— —	—
130	— —	—	0.109 $\pm$ 0.019	14.2
160	0.0274 $\pm$ 0.0042	4.38	0.103 $\pm$ 0.011	16.5
200	0.0198 $\pm$ 0.0030	3.96	— —	—
288	0.0243 $\pm$ 0.00061	7.00	— —	—

Mittelwert der  $E_1$ -bzw.  $E_4$ -Werte, die den  $t$ -Werten 1 bis mitsamt 100  $\sigma$  bzw. 1 bis mitsamt 40  $\sigma$  entsprechen:  $E_1 = 2.81 \pm 0.49$  bzw.  $E_4 = 6.44 \pm 0.43$  Lux  $\cdot$  Sigmen.

Die Zahlen der Kolumne der *absoluten Empfindungsschwellen* sind so erhalten, dass bei den in der Kolumne  $t$  angegebenen Reizzeiten von 1 bis 288  $\sigma$ , bei jeder Reizzeit 10 Bestimmungen (bei jeder Bestimmung 4 Lichtdarbietungen) der entsprechenden Lichtintensität ausgeführt wurden. Es wurde nach der Herstellungsmethode verfahren, wobei die Intensität in Schritten von 1 Grad der gebrauchten Blendenskala 5 mal erniedrigt und 5 mal erhöht wurde, bis im ersten Falle das Erlebnis eben verschwand,

im zweiten eben auftauchte. Es wurden in jedem Versuch 4 Kreiselrotationen, also 4 Belichtungen des Auges vorgenommen (in den Versuchen an den höheren Schwellen wurde ganz entsprechend verfahren). Aus der Tabelle ist deutlich ersichtlich, wie die Intensität (also Mittelwerte von 10 Bestimmungen; die mittleren Fehler von der Grössenordnung 10 %) bei Vergrösserung der Zeitdauer kleiner wird, so dass das Produkt der beiden Grössen  $L \cdot t$  ziemlich von gleicher Grösse ist, bis zu Reizdauern von etwa 100  $\sigma$ . Die von BLOCH, CHARPENTIER, v. KRIES und PIÉRON gefundene Regel bestätigt sich also auch hier (da der Durchmesser des Lichtkreises in unseren Versuchen 29.4 mm war und der Kreis von der Versuchsperson in 3 m Entfernung lag, entspricht dies einem Knotenpunktswinkel von 34', entsprechend dem grössten Wert in den Bestimmungen von v. KRIES). Wir nannten diese Regel die *Einschränkungsrelation* der absoluten Schwelle oder der Minimallichtbeobachtung und schlossen an ihr Gelten einige Betrachtungen über die Art der Lichtbeobachtung innerhalb kurzer Zeiten (Beobachtungsdauer = 100  $\sigma$ ) an (REENPÄÄ u. NIINI 1941). Die Produktgrösse  $L \cdot t$  hat den Mittelwert  $2.81 \pm 0.49$  Lux. Sigmen (berechnet für die Zeitwerte 1 bis 100  $\sigma$ ).

Bei längeren Zeitdauern des Reizes als 100  $\sigma$  ist die Lichtintensität relativ konstant; Mittelwert von  $L$  entsprechend den  $t$ -Werten 160, 200 u. 288  $\sigma$ ;  $0.0238 \pm 0.0027$  Lux.

In diesem Zusammenhang ist es von Interesse zu erfahren, bis zu welcher Zeitdauer der Reize die Lichterlebnisse *phänomenal als momentan* empfunden werden. Wir machten darum Versuche, bei denen der Vergleichsreiz immer 1  $\sigma$  dauerte, der Hauptreiz aber die verschiedenen, in der ersten Kolumne der Tabelle 1 angegebenen Zeitdauern von 1 bis 288  $\sigma$  annahm. Die Intensitäten der Reize waren natürlich, entsprechend den Mittelwerten der Tabelle, so angeordnet, dass sowohl Vergleichs- als Hauptreiz ein absolutes Schwellenerlebnis der Lichtintensität ergaben. Die Versuchsanzahl dieser Serie ist leider ungenügend; das Ergebnis scheint aber anzudeuten, dass erst ein Vergleichsreiz, der länger als 120  $\sigma$  ist, sicher auch phänomenal einen länger dauernden Eindruck macht als der Hauptreiz von 1  $\sigma$ . Die oberen zeitlichen Grenzen einerseits der Gültigkeit der Produktenregel der Konstanz des Produktes von Intensität und Zeitdauer des Reizes und andererseits der völligen Minimalität des Erlebnisses, also sowohl betreffs des Intensitätserlebnisses als des Zeiterlebnisses (völlig momentanes Erlebnis) scheinen also ungefähr an der selben Stelle zu liegen, bei etwa 100  $\sigma$ .

Tabelle 2.

Gegenseitige Abhängigkeit der Reizzeitdauer  $t$  (in Sigmen  $\sigma$ ) und der Reizlichtintensität  $L$  (in Lux) bei einer Empfindungsstärke, die äquivalent der 10. Intensitätsordnungsstelle bei Dauerbelichtung ist, an der Stelle 10, welche gleich der Schwelle mit der Ordnungszahl 5.34 ist, sowie an der Stelle 20, welche gleich der Schwelle mit der Ordnungszahl 8.43 ist. (Knotenpunktswinkel der Lichtkreise 34')

S t e l l e 1 0. (5.34 Schwelle)			S t e l l e 2 0. (8.43 Schwelle)	
$t$ $\sigma$	$L$ $L_{10}$	$L \cdot t = E_{10}$ $L_{10} \cdot \sigma$	$L$ $L_{20}$	$L \cdot t = E_{20}$ $L_{20} \cdot \sigma$
2	6.54 $\pm$ 0.42	13.1	12.06 $\pm$ 0.50	24.1
4	3.40 $\pm$ 0.21	13.6	6.81 $\pm$ 0.34	27.2
10	1.20 $\pm$ 0.14	12.0	3.15 $\pm$ 0.21	31.5
15	1.026 $\pm$ 0.078	15.4	2.17 $\pm$ 0.17	32.6
20	0.946 $\pm$ 0.058	19.9	1.71 $\pm$ 0.08	34.2
40	0.532 $\pm$ 0.014	21.3	0.808 $\pm$ 0.012	32.3
60	0.391 $\pm$ 0.020	23.5	0.631 $\pm$ 0.014	37.9
80	0.321 $\pm$ 0.019	25.7	0.524 $\pm$ 0.015	41.9
100	0.293 $\pm$ 0.012	29.3	0.472 $\pm$ 0.015	47.2
130	0.264 $\pm$ 0.027	34.3	0.430 $\pm$ 0.013	55.9
160	0.253 $\pm$ 0.012	40.5	0.402 $\pm$ 0.017	64.3

Mittelwert der  $E_{10}$ - bzw.  $E_{20}$ -Werte, die den  $t$ -Werten 2 bis mitsamt 40  $\sigma$  entsprechen:  $E_{10} = 15.88 \pm 0.74$  bzw.  $E_{20} = 30.32 \pm 1.15$  Lux. Sigmen.

Die Ergebnisse der Versuche mit *überschwelligen* Lichtreizen gehen aus den Tabellen 1 und 2 hervor. Diese Resultate sind so erhalten, dass der Versuchsperson mittels der einen von den beiden optischen Anordnungen ein Hauptreiz von 110  $\sigma$  Dauer und 0.0952, 0.238 bzw. 0.476 Lux, entsprechend den Intensitätsschwellenordnungszahlen 4, 10 und 20 (die angegebenen Lux-Werte sind 4, 10 und 20 mal grösser als der mittlere Intensitätswert bei langdauerndem Reiz an der absoluten Schwelle) und mittels der anderen optischen Anordnung ein Vergleichsreiz von den in den Tabellen angegebenen Zeitdauern und Intensitäten, mit Hilfe des rotierenden Kreisels immer abwechselnd dargeboten wurden. Die Grösse der Lichtkreise war hierbei dieselbe wie bei den Versuchen an der absoluten Schwelle. Der Abstand der Ränder der beiden Lichtkreise betrug 3 mm. Bei jeder Zeitdauer wurden, wie bei der absoluten Schwelle, zehn Einstellungen der Gleichheit der Intensität des Haupt- und der Vergleichsreize vorgenommen (die Werte

der Tabellen sind Mittelwerte dieser Bestimmungen). Da der Vergleichsreiz (Dauer 110  $\sigma$ ) eine Intensität von 0.0952, 0.238 bzw. 0.476 Lux hat, d. h. ein 4, 10 bzw. 20 mal stärkerer Reiz als die absolute Reizintensität bei Dauerbelichtung (besser: bei einer Belichtungsdauer, bei der die Zeit und die Intensität getrennt beobachtbar sind) ist, sind diese Werte in intensiver Hinsicht empfindungsäquivalent mit der 4- bzw. 10- bzw. 20-maligen absoluten Dauerreizschwelle.

Ans den Tabellen 1 und 2 ist ersichtlich, dass mitsteigender Dauer der Belichtung auch bei den überschwelligen Reizen bei gleichem Erlebnis die Intensität kleiner wird. An der Stelle 4 (Tabelle 1) scheint das Produkt  $L \cdot t$  bis zu Zeiten von etwa 40  $\sigma$  von relativ konstanter Grösse zu sein. Der Mittelwert der  $L \cdot t$ -Werte, die den  $t$ -Werten 1 bis mitsamt 40  $\sigma$  entsprechen, beträgt  $6.44 \pm 0.43$  Lux. Sigmen. Von der genannten Zeitdauer an scheint das Produkt zu steigen beginnen; die Intensität bei diesen längeren Zeitdauern bleibt dagegen relativ konstant und ihr Mittelwert beträgt entsprechend den Zeitdauern von 100 bis 160  $\sigma$ ,  $0.111 \pm 0.005$  Lux.

An der Stelle 10 (Tabelle 2) sehen wir, dass das Produkt  $L \cdot t$  eigentlich nur bis zu dem Zeitwert 15  $\sigma$  relativ konstant ist. Für die  $L$ -Werte finden wir dagegen, dass hier eine deutliche Verkleinerung dieser Werte entsprechend dem Steigen der Zeitwerte, bis zu Zeitdauern von 60  $\sigma$ , vor sich geht. Bei grösseren Zeitdauern ( $> 60 \sigma$ ) ist der  $L$ -Wert von relativ konstanter Grösse. Dementsprechend haben wir die den  $t$ -Werten von 2 bis mitsamt 40  $\sigma$  entsprechenden Mittelwerte der Produkte  $L \cdot t$  berechnet. An der Stelle 10 beträgt dieser Wert  $15.88 \pm 0.74$  Lux. Sigmen.

Und schliesslich sehen wir, dass an der Stelle 20 (Tabelle 2) das Produkt  $L \cdot t$  überhaupt nicht gut konstant ist. Bis zu Zeitdauern von 40  $\sigma$  ist es jedoch von relativ konstanter Grösse und dementsprechend haben wir den den  $t$ -Werten 2 bis mitsamt 40  $\sigma$  entsprechenden Mittelwert berechnet; er beträgt  $30.32 \pm 1.15$  Lux. Sigmen.

Ein Vergleich der  $L \cdot t$ -Werte an der absoluten, also der 1. Schwelle, sowie an der 4, 10 und 20 mal dieser Schwelle entsprechenden Reizen zeigt, dass es bei ihnen allen einen Bereich gibt, in dem dieses Produkt relativ konstant ist. An der absoluten Schwelle ist dieser Bereich jedoch zeitlich ausgedehnter als bei den höheren Reizwerten. Dagegen ist derjenige Bereich, innerhalb dessen die Zeitdauer und die Intensität überhaupt eine gegen-



seitige Abhängigkeit zeigen, bei allen untersuchten Reizwerten zeitlich ziemlich ausgedehnt. An der absoluten Schwelle reicht dieser Bereich bis zu der Zeitdauer von 100—120  $\sigma$ , bei den der 4-maligen Dauerschwelle (Schwelle der Intensität bei lange dauernder Reizzeit) entsprechenden Reizen reicht er ebenfalls bis zu 100  $\sigma$ , und bei den entsprechend 10- und 20-maligen Reizen scheint er noch nicht deutlich bei der Dauer von 160  $\sigma$  erreicht zu sein.

Entsprechend wie an der absoluten Schwelle haben wir auch an den Stellen 2, 4 und 10 Versuche angestellt, um zu erfahren, bis zu welchen Zeitdauern die Erlebnisse bei diesen Stellen phänomenal als momentan empfunden werden. An den Stellen 4 und 10 sind etwas grössere Versuchsreihen angestellt worden mit einem Hauptreiz von 1 bzw. 2  $\sigma$  Dauer und mit Vergleichsreizen von 1 bis zu 100 bzw. 160  $\sigma$ . Das Ergebnis ist, dass an der Stelle 4 von Zeitdauern von 20  $\sigma$  an die Mehrzahl der Vergleichsreize als längerdauernd als der Hauptreiz von 1  $\sigma$  empfunden werden. Unterhalb dieser Zeitgrenze wird die Mehrzahl der Vergleichsreize als mit dem Hauptreiz gleichlange dauernd empfunden. An der Stelle 4 scheint sich also die *phänomenale Momentanität* der Reize bis zu etwa 20  $\sigma$  zu erstrecken. An der Stelle 10 ist das Ergebnis ganz entsprechend angestellter Versuche, dass diese Grenze hier bei 15  $\sigma$  liegt. An der Stelle 20 haben wir als entsprechenden Zeitwert 30  $\sigma$  erhalten.

Zusammenfassend scheint die Zeitgrenze der phänomenalen Momentanität an den gebrauchten höheren Schwellen bei 20—30  $\sigma$  zu liegen, also jedenfalls deutlich unterhalb des entsprechenden Wertes an der absoluten Schwelle (100  $\sigma$ ). An den höheren Schwellen ist auch der Gültigkeitsbereich der Produktenregel  $L \cdot t = \text{Konstante}$  zeitlich begrenzter als an der absoluten Schwelle. An dieser letzteren reichte dieser Bereich zeitlich bis zu 100  $\sigma$ , entsprechend der Gültigkeit der phänomenalen Momentanität der Empfindung. Bei den überschwelligen Reizen gilt die Produktenregel bis zu etwa 40  $\sigma$ , die phänomenale Momentanität bis zu etwa 20—30  $\sigma$ ; also auch eine gewisse Übereinstimmung.

Zusammenfassend dürfte man also sagen können, dass *wenn es sich um in phänomenaler Hinsicht völlig minimale Empfindungen handelt (also wenn sowohl die zeitliche wie die intensive Dimension minimal ist), die Produktenregel genau gilt*. Wenn dagegen die phänomenale Zeitdimension wohl minimal, die Intensitätsdimension dagegen überschwellig ist, gilt diese Regel nur annäherungs-

weise und sie ist zeitlich beschränkt. Ausserhalb des momentanen Zeitgebietes, wo also die Zeitdauer und die Intensität zum Teil als gesonderte Phänomenalitäten erlebt werden können, gilt die Produktenregel nicht mehr genau; entsprechend der gesonderten Phänomenalität dieser beiden Dimensionen bekommen hier auch ihre begrifflichen Reizentsprechungen eine selbständige Bedeutung.

Wenn wir die Mittelwerte der in den Tabellen 1 und 2 angegebenen, bei den Momentanbeobachtungsversuchen erhaltenen konstanten Produkte  $L \cdot t = E_n$  (wobei der Index  $n$  die »Stelle«, also den Wert 1, 4, 10 bzw. 20 angibt) durch die Stellen-Werte  $n$  oder durch die mit ihnen proportionalen Intensitätswerte der entsprechenden Dauerbeobachtung  $L_n$ , welche in den Versuchen als Hauptreize dienten, dividieren, erhalten wir die, in der Tabelle 3 angegebenen Werte  $\frac{E_n}{n}$  bzw.  $\frac{E_n}{L_n}$ . Diese Werte sind, den ersten ausgenommen, ersichtlich alle ziemlich gleichgross. Als empirisches Ergebnis erhält man also, dass *gleichgrossen Zunahmen der Intensitäten der Dauerreize immer gleichgrosse Zunahmen der die gleichen Erlebnisse bedingenden Energiegrössen der Momentanreize entsprechen.*

Tabelle 3.

Stelle $n$	$L_n$	$E_n$	$\Delta E_n$	$\frac{E_n}{n}$ $L_{ux} \cdot \sigma$ pro »Stelle«	$\frac{E_n}{L_n}$	Schwellenord- nungszahl berechnet mittels dem Wert	
						$2 \cdot S_{50}$ $m_1$	$2 \cdot \Delta L_2$ $m_2$
1	0.0238	$2.81 \pm 0.49$		2.81	118.1	1	1
4	0.0954	$6.44 \pm 0.43$		1.61	67.6	3.17	2.8
10	0.238	$15.88 \pm 0.74$		1.59	66.7	6.3	5.84
20	0.476	$30.32 \pm 1.15$		1.52	63.7	10.0	8.43

Mittelwert  
der drei  
unteren  
Werte

$$\frac{E_n}{L_n} = 66.0$$

Dieses überraschende Ergebnis bedingt auch zugleich, dass die aus diesen Werten zu berechnende Energiezunahme des Momen-

tanreizes berechnet pro *Dauerintensitätsschwelle* nicht konstant sein kann, denn die Dauerintensitätsschwellen nehmen bekanntlich mit der Intensität allmählich zu. Die Berechnung der Energiewerte pro Schwelle kann aber auch in anderer Weise ausgeführt werden, worauf aber hier nicht eingegangen werden soll.

### Über die Intensitätsunterschiedsschwellen mit den niedrigsten Ordnungszahlen bei Dauerbelichtung.

Die absolute Schwelle kann als die 1. Schwelle angesetzt werden. Der Intensitätswert dieser 1. Schwelle ist bei Dauerbelichtung 0.0238 Lux. Wenn die 2., 3. usw. Schwelle, also die folgenden relativen Schwellen, die Unterschiedsschwellen bei Dauerbelichtung, von der gleichen Grösse wären, wäre der dem Lux-Wert von 0.238 entsprechende Dauerreiz 10 mal grösser als der absolute Reiz und die ihm äquivalenten Reize wären 10-malige Schwellenreize. Nun ist aber die Unterschiedsschwelle bei *Dauerbelichtung* an der Intensitätsstelle 0.238 Lux nicht 0.0238, sondern sie kann gleich 0.028 oder 0.041 Lux gesetzt werden je nachdem ob die Unterschiedsschwelle gleich dem doppelten  $S_{50}$ -Wert (derjenige Wert, welcher die Grenze zwischen den zu 50 Prozent gleichen und den zu 50 Prozent ungleichen Urteilen entspricht) oder gleich der doppelten mittleren quadratischen Abweichung  $\Delta L_2$  gesetzt wird (das Ausführen der Unterschiedsschwellenbestimmungen wird im folgenden beschrieben). Die beiliegende Abb. 2 veranschaulicht, wie sich die Verteilungskurven der aufeinander

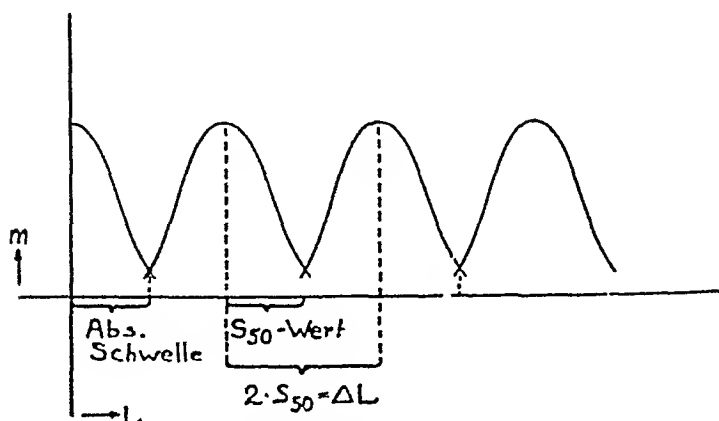


Abb. 2. Schematische Darstellung der Verteilungskurven (absolute Schwelle und Unterschiedsschwellen) der richtigen und falschen Fälle an verschiedenen Stellen (Intensitäten  $I$ ) des Reizes. Näheres im Text.

folgenden Schwellen ungefähr zueinander verhalten. Das Streuungsmass  $S_{50}$  ist in die Abbildung eingezeichnet. Augenscheinlich liegen die Kuppen der nacheinanderfolgenden Verteilungskurven im Abstand von  $2 \cdot S_{50}$  von einander. Nun wissen wir nicht, welche Form die Verteilungskurven in unserem Fall haben. Demgemäss berechnen wir auch aus unseren im folgenden zu besprechenden Versuchen, sowohl den Wert  $S_{50}$  als den Wert  $\Delta L_2$ .

Intensitätsunterschiedsschwellenbestimmungen bei Dauerbelichtung sind denn auch seit langer Zeit in grosser Anzahl von vielen Forschern ausgeführt worden (siehe STEINHARDT 1937). Es fehlen aber in der Literatur Angaben über die Grösse derjenigen Intensitätsunterschiedsschwellen (gemessen als Lichtintensitäten), welche ganz in der Nähe der absoluten Schwelle, der 1. Unterschiedsschwelle liegen. Wir wissen, dass die Grösse der Intensitätsunterschiedsschwelle bei Dauerbelichtung mit der Intensität der Grundbelichtung zunimmt (STEINHARDT) und man hat Formeln aufstellen können, die den Zusammenhang zwischen den Intensitäten der Schwelle und der Grundbelichtung ausdrücken (siehe auch REENPÄÄ, 1946). Jedenfalls dürfte es von Interesse sein, das zu vermutende Ansteigen der allerniedrigsten Intensitätsunterschiedsschwellen genauer zu bestimmen. Und besonders wollten wir noch erfahren, wie sich die allerniedrigsten Unterschiedsschwellen in ihrer Grösse zu derjenigen der 1. Schwelle, der absoluten Schwelle verhalten.

### Die Methode der Bestimmung der ersten Unterschiedsschwellen.

Die Anordnung war die im vorigen beschriebene, aber für genauere Intensitätsregulierung ergänzt, da diese mit den Blenden (Abb. 1) nicht fein genug zu erzielen war. An der Stelle K der Anordnung (optische Bank des Projektionssystems A) war ein sehr spitzes Rauchkeilglas angebracht, welches mittels einer Schraube senkrecht zur Projektionsrichtung des Lichtes bewegt werden konnte. Durch verschiedene Einstellung des Keilglases konnte die Intensität des Projektionskreises am Schirm (der Projektion der Lampe P, A) dann sehr genau abgestuft werden. Der andere Lichtkreis am Schirm, dessen Intensität konstant gehalten wurde, wurde von der optischen Anordnung B projiziert; der Vergleich war also ein simultaner. Die Grösse der Kreise war dieselbe wie bei den im vorigen beschriebenen Versuchen. Dasselbe gilt für den Abstand der Lichtkreise von einander. Die Absorption der benutzten zwei Rauchkeilgläser wurde unter Zugrundelegung der Methode der richtigen und falschen Fälle an fünf bzw. drei Stellen genau bestimmt und die erhaltenen Werte graphisch aufgezeichnet und durch

eine Kurve verbunden. Die an den verschiedenen Stellen der Keilgläser stattfindende Lichtabsorption wurde aus diesen Eichkurven abgelesen.

Die *Unterschiedsschwellen* wurden dann folgendermassen bestimmt. Die Intensität der Projektionsanordnung B (des Hauptreizes) wurde auf die »Stelle« 2 eingestellt. Die Intensität (am Schirm) dieser Stelle ist, wie im vorigen erwähnt, 0.048 Lux (sie entspricht aber nicht der 2. Unterschiedsschwelle, sondern der Schwelle mit der Ordnungszahl 1.48, wenn die absolute Schwelle als die Schwelle mit der Ordnungszahl 1. angesetzt wird), wenn wir eine lineare Abhängigkeit zwischen den Ordnungszahlen dieser Unterschiedsschwellen und den entsprechenden Stellenzahlen annehmen, was wohl berechtigt ist, da es sich um schwellemnässige Unterschiede handelt. Durch Probieren werden dann diejenigen Grenzwerte des Keilglases, das vor der Projektionsanordnung A steht, ausfindig gemacht, innerhalb welcher sich die im Vergleich zum Kreis B sicher über- und unterschwelligen Werte des Lichtkreises A befinden. Mit etwa 20 verschiedenen, innerhalb dieser Grenzen befindlichen Intensitätswerten wurden dann, unter Benutzung der Methode der richtigen und falschen Fälle (wobei die Urteile +, — und = abgegeben wurden) Bestimmungen ausgeführt. Bei jeder Intensität wurden 20 Urteile abgegeben.

Als *Ergebnis* dieser Bestimmungen finden wir, dass an der Stelle 2., also der Schwelle 1.48, derjenige Intensitätsbereich, welcher den 50-prozentigen Gleichheitswerten entspricht, 0.0292 Lux beträgt. Die Hälfte dieses Wertes, der sog.  $S_{50}$ -Wert, ist also  $S_{50} = 0.015$  Lux. Da die quadratische mittlere Abweichung  $\Delta L_2 = 3/2$  dieses Wertes ist, haben wir an der Stelle 2.,  $\Delta L_2 = 0.022$  Lux. Entsprechend erhalten wir an der Stelle 10., welche der Schwelle mit der Ordnungszahl 5.34 entspricht;  $S_{50} = 0.028$  bzw.  $\Delta L_2 = 0.041$  Lux.

In der unmittelbaren Nähe der 1., der absoluten Schwelle, deren Grösse bei Dauerbelichtung wir im vorigen zu 0.024 Lux bestimmten, an der Schwelle 1.48, beträgt die mittlere quadratische Abweichung also 0.022 und der  $S_{50}$ -Wert 0.015 Lux. Als Grösse der Unterschiedsschwelle müssen wir jedoch den doppelten  $S_{50}$ -Wert bzw. den doppelten quadratischen Fehler setzen. Die Reizunterschiedsschwelle ist die implikative Entsprechung der Empfindungsunterschiedsschwelle (s. RENQVIST-REENPÄÄ, 1936). Die Anzahl der Schritte in der Reihe der nacheinander folgenden Empfindungsunterschiede wird, wie im vorigen dargelegt wurde (s. die Abb. 2), durch die Entfernung der einzelnen, jedem Reizwert entsprechenden Verteilungskurven angegeben. Und diese Entfernung setzen wir also gleich entweder  $2 \cdot S_{50}$  oder  $2 \cdot \Delta L_2$ ; eine bestimmte Wahl kann nur durch Definition erfolgen. Die

Grösse der Unterchiedsschwelle an der Schwelle mit der Ordnungszahl 1.48 wäre hiernach  $0.029 (= 2 \cdot S_{50})$  bzw.  $0.044 (= 2 \cdot A L_2)$  Lux.

Die Grösse der absoluten Schwelle wurde mittels der Herstellungsmethode bestimmt, wobei von 10 einzelnen Bestimmungen der Mittelwert genommen wurde. Eine jede einzelne Bestimmung dürfte hierbei wahrrscheinlichkeitsgemäss einen Wert geben, der an der  $S_{50}$ -Grenze liegt. Wenn dem so ist, dürften die mittels der Methoden der 50 prozentigen Verteilung und der Herstellung gefundenen Werte, als nahe bei einander liegend, mit einander verglichen werden können. Die Methode der Bestimmung der absoluten, der 1. Schwelle ergab aber einen dem *einfachen*  $S_{50}$ -Wert der Unterchiedsschwelle an der Schwelle mit der Ordnungszahl 1.48 entsprechenden Wert; die Werte sind 0.024 bzw. 0.028 Lux. Dies ist auch in der Abb. 2 angedeutet. Unser Wert der absoluten Schwelle 0.024 Lux repräsentiert hiernach den ersten Halbschritt in der Reihe der nacheinander folgenden Unterschiedsschwellen. An der Schwelle mit der Ordnungszahl 1.48 ist der Vollschrirt dann, wie dargelegt, gleich 0.029 Lux, d. h. der Halbschritt ist an dieser, sehr nahe der absoluten Schwelle liegenden Stelle etwas kleiner als der allererste Halbschritt; der 1. Halbschritt (d. h. die absolute Schwelle) ist 0.024 Lux, der Halbschritt an der Stelle 1.48 ist 0.015 Lux.

Wie die Bestimmungen ergaben, ist der entsprechende Halbschritt an der Schwelle 5.34 dann 0.028 Lux. Die Werte der Halbschritte scheinen also von der Schwelle 1.48 bis zur Schwelle 5.34 grösser geworden zu sein. Auf Grund alles Vorgenannten scheint also *die absolute Schwelle (in Lux) etwas grösser zu sein als die allerersten, in ihrer Nachbarschaft liegenden Reizunterschied-(Halb-)Schwellen*, welche letztere am natürlichsten mit ihr zu vergleichen sind. Die Grösse der Unterschiedsschwellen dürfte aber dann von Schwelle zu Schwelle steigen, um etwa an der 4. bis 5. Schwelle die Grösse der absoluten Schwelle zu erreichen und um dann fortwährend immer grösser zu werden.

Bei grösseren Grundreizen werden dann die Grössen der Unterschiedsschwellenreize, gemessen als physikalische Lichtintensitäten, immer grösser; das Verhältnis dieser weit überschwelligen Unterschiedsschwellenreize zu den Grundreizen haben die Untersuchungen von HECHT (1935) sowie STEINHARDT klargelegt.

Schliesslich soll hier darauf hingewiesen werden, dass das Problem des Zusammenhangs einerseits des Verhältnisses des Unter-

schieds- und Grundreizes sowie andererseits der Wahl der zu verwendenden physikalischen Reizgrösse von einem von uns in anderem Zusammenhang behandelt worden ist (REENPÄÄ, 1946).

### Untersuchung der gegenseitigen Abhängigkeit der Reizgrössen Lichtintensität und Flächengrösse.

Diese Untersuchung wurde sowohl an der absoluten Schwelle wie an zwei überschwelligen Stellen, den Stellen 4 und 10 entsprechend den Schwellen mit den Ordnungszahlen 2.8 und 5.34 vorgenommen. Das Interesse bei diesen Versuchen richtete sich besonders auf die Untersuchung der Grenzen der Gültigkeit der von ASHER, WALD, CROZIER u. a. gefundenen, von uns als Einschränkungrelation bezeichneten Regel.

Die *Versuchsanordnung* ist die eingangs beschriebene (Abb. 1). Die *Grösse der Lichtfläche* wurde also mittels der Blenden ( $I_3$ ) eingestellt. Da bei diesen Versuchen besonders aber auch sehr kleine Lichtflächen zur Verwendung kommen, wurden an der Apparatur an der Stelle  $I_3$  auch ganz enge Lochblenden verwendet. Die *Intensität* wurde in der im vorigen beschriebenen Weise reguliert. Alle Versuche waren Dauerversuche, deren Reizzeitdauer 110  $\sigma$  war. Wie bei den vorher beschriebenen, wurden auch bei diesen Versuchen pro Versuch 4 nacheinanderfolgende Reize gegeben.

Die *Ergebnisse* der Bestimmungen an der absoluten Schwelle sind in Tabelle 4 zusammengestellt. Ersichtlich bewegen sich die verwendeten Flächengrössen ( $f$ ) in sehr weiten Grenzen, von 0.665 qmm bis 1,255 qmm, entsprechend Gesichtswinkeln von 1.01 bis 44.0 Bogenminuten. Die entsprechende Lichtintensität ( $L$ ) wird entsprechend dem Steigen der Flächengrösse immer kleiner, anfangs rapide, dann langsamer, so dass das Produkt  $L \cdot f$  anfangs ziemlich konstant bleibt, bei grösseren Flächen aber zu wachsen beginnt. Wir sehen, dass das Produkt  $L \cdot f$  bis zum Knotenpunktswinkel  $2.87'$ , entsprechend der Flächengrösse von etwa 5.35 qmm, von ziemlich konstanter Grösse ist, mit dem Mittelwert  $4.02 \pm 0.37$  Lux. qmm. Die Einschränkungrelation  $L \cdot f = \text{Konstante}$ , also eine der im vorigen beschriebenen Einschränkung in zeitlicher Beziehung entsprechende Relation betreffs der Fläche, gilt also bei der absoluten Schwelle von ganz kleinen Lichtflächen bis zu einem Grenzwert, welcher einem Knotenpunktswinkel von etwas über  $2'$  entspricht. Das Ergebnis stimmt also mit demjenigen von ASHER überein.

Tabelle 4.

Gegenseitige Abhängigkeit der Reizfläche  $f$  (in qmm) und der Reizlichtintensität  $L$  (in Lux) an der absoluten Empfindungsschwelle, der Stelle 1.  $d$  = Diameter des Lichtkreises in mm (in 3 m Entfernung von der Vp.),  $\delta$  = entsprechender Knotenpunktswinkel in Bogenminuten,  $f$  = entsprechende Fläche des Lichtkreises in qmm.

d mm	$\delta$ Bogen- minuten	f qmm	L Lux	$L \cdot f$ Lux · qmm
0.92	1.01	0.665	6.33	4.21
1.10	1.21	0.950	3.76	3.57
1.36	1.50	1.45	3.22	4.67
2.07	2.28	3.35	1.06	3.55
2.61	2.87	5.35	0.770	4.12
4.6	5.1	16.6	0.295	4.90
6.0	6.6	28.3	0.235	6.65
8.1	8.9	51.5	0.122	6.28
16.1	17.7	203.	0.0426	8.65
25.1	27.6	495.	0.0296	14.65
40.0	44.0	1,255.	0.0213	26.7

Mittelwert der  $L \cdot f$ -Werte, die den  $f$ -Werten 0.665 bis mitsamt 5.35 qmm entsprechen:  $L \cdot f = 4.02 \pm 0.37$  Lux · qmm.

An der Stelle 4, also an der Schwelle mit der Ordnungszahl 2.8 wurden entsprechende Versuche angestellt, deren Ergebnis in der Tabelle 5 zusammengestellt ist. Der Hauptreiz hatte hier eine Flächengrösse von 5.35 qmm sowie eine Intensität, welche der Schwelle 2.8 bei Dauerbelichtung entspricht (3.08 Lux). Bei den Vergleichsreizen von verschiedener Flächengrösse wurden dann die dem Hauptreiz erlebnisäquivalenten Intensitäten aufgesucht; hier wie in allen unseren Versuchen wurden zehn Reizeinstellungen verwendet, deren Mittelwert die Tabellen enthalten. Das Produkt  $L \cdot f$  scheint hier nicht besonders gut konstant zu sein, auch nicht bei den kleinsten Lichtflächen. Das Produkt beginnt aber, von der Flächengrösse 3.35—7.54 qmm an, fortwährend grösser zu werden, entsprechend einem Knotenpunktswinkel von 2' bis 3'. An der Schwelle 2.8 dürfte das Ergebnis also, obwohl weniger überzeugend, demjenigen an der absoluten, der 1. Schwelle entsprechen.

In der Tabelle 6 sind schliesslich die an der Stelle 10, d. h. der Schwelle 5.34 angestellten Versuche zusammengestellt. Auch



Tabelle 5.

Gegenseitige Abhängigkeit der Reizfläche  $f$  (in qmm) und der Reizlichtintensität  $L$  (in Lux) an der Schwelle mit der Ordnungszahl 2.8, der Stelle 4. Die Bezeichnungen sind dieselben wie in der Tabelle 4.

d mm	$\delta$ Bogen- minuten	f qmm	L Lux	$L \cdot f$ Lux · qmm
1.10	1.21	0.960	14.4	13.7
1.36	1.50	1.45	12.3	17.7
2.07	2.28	3.35	5.30	17.8
3.1	3.41	7.54	3.14	23.7
4.6	5.1	16.6	1.75	29.1
6.0	6.6	28.3	1.22	34.5
8.1	8.9	51.5	1.28	65.9
16.1	17.7	203.	0.96	195.
25.1	27.6	495.	0.85	421.
40.0	44.0	1,255.	1.04	1,305.

Mittelwert der  $L \cdot f$ -Werte, die den  $f$ -Werten 0.665 bis mitsamt 7.54 qmm entsprechen:  $L \cdot f = 18.2 \pm 2.6$  Lux · qmm.

hier hatte der Hauptreiz eine Flächengrösse von 5.35 qmm und eine Intensität von 7.70 Lux, entsprechend der Schwellenzahl 5.34. An dieser Schwelle gilt die Einschränkungrelation  $L \cdot f =$  Konstante ziemlich gut bis zu der Reizfläche von 7.54 qmm, entsprechend einem Knotenpunktswinkel von  $3.41'$ . Es ist befremdend, dass der Wert des Produktes  $L \cdot f$  an der Schwelle 5.34 kleiner als an der Schwelle 2.8 ist. Die Ursache dieses Verhaltens dürfte daran liegen, dass die Versuche an der Schwelle 2.8 (wie auch an der absoluten Schwelle) unter Verwendung eines schwach fluoreszierenden Fixationspunktes, die Versuche an der Schwelle 5.34 dagegen, wegen der Stärke des Reizes, ohne Fixationspunkt ausgeführt wurden.

Zusammenfassend sehen wir aus den Tabellen 4, 5 und 6, dass die Einschränkungrelation der Fläche bei den Schwellen mit den Ordnungszahlen 1. (der absoluten Schwelle), 2.8 und 5.3 bis zu Flächengrössen gilt, deren Knotenpunktswinkel etwa  $2'$  bis  $3'$  sind.

Dieses Ergebnis scheint im Zusammenhang mit einem Ergebnis von TONNER (1943) über die Grösse der sog. Empfindungsfläche eines Lichtpunktes zu stehen. TONNER bezeichnet als den Durchmesser der Empfindungsfläche diejenige Strecke, die ein

Tabelle 6.

Gegenseitige Abhängigkeit der Reizfläche  $f$  (in qmm) und der Reizlichtintensität  $L$  (in Lux) an der Schwelle mit der Ordnungszahl 5.34, der Stelle 10. Die Bezeichnungen sind dieselben wie in der Tabelle 4.

d mm	$\delta$ Bogen- minuten	f qmm	L Lux	$L \cdot f$ Lux · qmm
0.92	1.01	0.665	11.24	7.48
1.10	1.21	0.950	7.80	7.41
1.86	1.50	1.45	6.48	9.40
2.07	2.28	3.35	2.35	7.87
2.61	2.87	5.35	1.78	9.26
3.1	3.41	7.54	1.18	8.52
4.8	4.7	14.6	0.927	13.5
5.8	6.4	26.4	0.540	14.3
8.8	9.1	54.0	0.850	18.9
10.6	11.7	88.4	0.832	29.8
16.0	17.6	201.	0.801	60.5
21.2	23.8	353.	0.282	99.5
26.7	29.4	560.	0.262	147.
29.4	32.8	680.	0.261	178.

Mittelwert der  $L \cdot f$ -Werte, die den  $f$ -Werten 0.665 bis mitsamt 7.54 qmm entsprechen:  $L \cdot f = 8.32 \pm 0.737$  Lux · qmm.

leuchtender kleiner Kreis bewegt werden muss, um phänomenal um seinen eigenen Durchmesser weiter verschoben zu erscheinen. Betreffs der Bestimmung dieser Grösse verweisen wir auf die Abhandlung von TONNER (seine 2. Mitteilung). Die Empfindungsfläche von TONNER ist also die Grösse der phänomenalen Minimalfläche, diejenige Fläche, die als eine Ganzheit empfunden wird, und sie entspricht bei optimalen Unterscheidungsverhältnissen einem Knotenpunktswinkel von 72—73". Die Grösse der Empfindungsfläche zeigt eine Abhängigkeit von der Punkthelligkeit. Bei schwach belichteten Punkten war die Grösse der Empfindungsfläche grösser (120"), bei mittleren Helligkeiten war sie von der Grösse 72—73". Da der Zapfendurchmesser in der Fovea (im Mittel  $2.5 \mu$ ) einem Knotenpunktswinkel von 30" entspricht, beträgt der Durchmesser der Empfindungsfläche also 2 bis 3 Zapfendurchmesser. Auf Grund dieses Befundes sowie anderer, sehr aufschlussreicher Ergebnisse gibt TONNER ein Schema der möglichen Empfindungsfläche der Fovea. Nach ihm dürfte sie aus

einem Zentralzapfen und 6 Kranzzapfen bestehen. Die kleinste Empfindungsfläche eines punktförmigen Objektes beruhe also auf der Erregung von mehreren, wahrscheinlich 7 Zapfen. Die Wirkungseinheit der Sehsphäre wäre diese Zapfengruppe.

Unsere Bestimmungen der Grenze der Gültigkeit der Einschränkungrelation der Flächen ergaben als Obergrenze eine Fläche, die einem Knotenpunktswinkel von etwa  $2$  bis  $3' = 120$  bis  $180''$  entspricht. Wir sehen, dass die Obergrenze des Knotenpunktswinkels der Flächengrösse der Gültigkeit der Einschränkungrelation etwas grösser ist als die Grösse des entsprechenden Winkelmasses der optimalen Empfindungsfläche von TONNER. Unsere Bestimmungen sind bei niedrigeren Lichtintensitäten ausgeführt worden, die TONNER'schen an stärkeren. Unter Berücksichtigung dessen, dass die Empfindungsfläche mit sinkender Lichtintensität steigt, dürfte man der grössenordnungsgemässen Übereinstimmung zwischen Umfang der Empfindungsfläche und Geltungsbereich der Einschränkungrelation eine der zeitlichen Einschränkungrelation ähnliche Interpretation geben können.

Die Versuche, bei denen Intensität und Zeitdauer des Reizes variiert wurden, ergaben als Resultat, dass bei phänomenal in allen Beziehungen minimalen Empfindungen, also betreffs aller phänomenalen Dimensionen der absoluten Empfindungen, aber, obwohl weniger genau, auch betreffs der Intensitätsdimension überschwelligem aber betreffs der Zeitdimension phänomenal momentanen Empfindungen, die Reizproduktenregel Intensität mal Zeit gleich einer Konstante ( $L \cdot t = \text{Konstante}$ ) gilt. Es handelt sich hier also um in zeitlicher Beziehung *phänomenal-momentane Erlebnisse*. Ausserhalb des momentanen Zeitgebietes, wo die Zeitdauer und die Intensität zum Teil als gesonderte Phänomenalitäten erlebt werden können, gilt die Produktenregel nicht mehr.

Die Versuche über die gegenseitige Abhängigkeit der Reizgrössen Intensität und Flächengrösse haben ein in einer vielleicht bedeutungsvollen Beziehung gleichartiges Ergebnis gezeitigt. Sie ergaben, dass *bei sowohl betreffs der phänomenalen Flächengrösse als der Intensität minimalen, wie betreffs nur der erstgenannten Phänomenalität minimalen Reizen, die Produktenregel Intensität mal Flächengrösse gleich einer Konstante ( $L \cdot f = \text{Konstante}$ ) gilt. Hier handelt es sich also um in räumlicher (in flächengemässer) Beziehung phänomenal-punktueller Erlebnisse*. Und auch hier gilt die Produktenregel nicht mehr ausserhalb dieses punktuellen Flächengebietes, also ausserhalb der Fläche des Empfindungs-



Einschränkungsrelation), was ein Hinweis darauf ist, wo und in welcher Weise die Genese, das Aufkommen der die Phänomenalitäten, die Beobachtungen beschreibenden begrifflichen (Reiz-) Grössen zu suchen ist.

### Zusammenfassung.

1. Auf dem Gebiete des Gesichtes haben BLOCH, CHARPENTIER, v. KRIES und PIÉRON gezeigt, dass in den Reizausdrücken, die der phänomenalen absoluten Empfindungsschwelle entsprechen, das Produkt der Reizgrössen Lichtintensität  $L$  (welche eine Grösse von der Dimension einer Leistung ist) und Zeitdauer  $t$  eine Konstante ist:  $L \cdot t = E_1$ , wobei  $E_1$  die konstante Energie des Minimalreizes bedeutet. Die Regel gilt genau bis zu Zeitdauern von 100 bis 120  $\sigma$ .

2. Eine der obigen Relation ähnliche Beziehung in betreff der Reizgrössen Lichtintensität  $L$  und Flächengrösse  $f$  hat ASHER gefunden. Entsprechend der phänomenalen absoluten Empfindungsschwelle gilt die Beziehung  $L \cdot f = L_1$ , wobei  $L_1$  eine konstante Grösse bezeichnet, die von der physikalischen Dimension einer Leistung ist. Diese Beziehung gilt nur für Flächen, die kleineren Knotenpunkts winkeln als etwa  $2'$  entsprechen.

3. Wir untersuchten erneut die gegenseitige Abhängigkeit der Reizgrössen Intensität und Zeit bzw. Fläche entsprechend der absoluten sowie einiger *überschwelliger* Empfindungsstellen. Besonders wurde bei unserer Untersuchung *den Grenzen* der Gültigkeit der Regeln Aufmerksamkeit geschenkt.

4. Das *Ergebnis* der Versuche betreffs der Reizgrössen Lichtintensität und Zeitdauer bestätigte die Gültigkeit der Produktenregel  $L \cdot t = E_1$  entsprechend der absoluten Empfindungsschwelle, bis zu Reizzeitdauern von 100  $\sigma$ . Bei *überschwelligen* Empfindungen gilt entsprechend den Schwellen mit den Ordnungszahlen 2.8, 5.34 und 8.43 näherungsweise auch eine Produktenregel:  $L \cdot t = E_n$ , wobei  $E_n$  die der  $n$ -ten Schwelle entsprechende Reizenergie bedeutet. Die Zeitgrenze des Geltens dieser Regeln ist etwa 40  $\sigma$ .

5. Es wurden Versuche angestellt, um zu erfahren bis zu welcher Zeitdauer der Reize die Lichtempfindungen *phänomenal als momentan* empfunden werden. An der absoluten Empfindungsschwelle erstreckt sich dieses Gebiet bis zu Zeitdauern von 120  $\sigma$ , bei den überschwelligen Empfindungen bis zu 20—30  $\sigma$ . Bei allen Schwellen scheinen also die oberen zeitlichen Grenzen einerseits der Gültigkeit der Produktenregeln und andererseits von der

phänomenalen Momentanität, ungefähr an derselben Zeit-Stelle zu liegen.

6. Das Ergebnis der Untersuchung der Abhängigkeit der Reizgrössen Intensität und Flächengrösse war eine Bestätigung des ASHER'schen Befundes vom Gelten der Produktenregel  $L \cdot f = \text{Konstante}$ , entsprechend der *absoluten Empfindungsschwelle* und bis zu Flächengrössen die einem Knotenpunktswinkel von  $2-3'$  entsprechen. An den untersuchten überschwelligen Stellen mit den Ordnungszahlen 2.8 und 5.34 gelten analoge Produktenregeln und auch hier bis zu einer Flächengrösse von derselben Grösse.

7. TONNER bezeichnet als Empfindungsfläche die Grösse derjenigen Fläche, die der phänomenalen Punktualität entspricht. Sie entspricht bei optimalen Unterscheidungsverhältnissen einem Knotenpunktswinkel von  $72-73''$ . Unsere Bestimmung der Grenze der Gültigkeit der Produktenregel der Fläche ergab als Obergrenze eine einem Knotenpunktswinkel von  $2-3'$  entsprechende Fläche. Da die Empfindungsfläche mit sinkender Lichtintensität grösser wird und da unsere Versuche mit kleineren Lichtintensitäten als diejenigen der Bestimmung der optimalen Empfindungsfläche ausgeführt sind, dürfte die Produktenregel nur dann gelten, wenn es sich um in räumlicher Beziehung *phänomenal punktuelle* Erlebnisse handelt.

8. Die Zusammenfassung der beiden Produktenregeln, die allgemeine Einschränkungrelation  $L \cdot f \cdot t = \text{Konstante}$ , welche genau nur dann gilt, wenn es sich um phänomenal in *allen* Beziehungen minimale Erlebnisse handelt, gibt an, dass die das Erlebnis begrifflich wiedergebenden Reizgrössen der Lichtintensität, der Flächengrösse und der Zeit einander gemäss der Produktenregel *linear* einschränken. Auf die Bedeutung dieses phänomenal-funktionellen Ausdrucks betreffs der Genese und der Bedeutung der die Phänomenalitäten, die Beobachtungen beschreibenden begrifflichen (Reiz-)Grössen wird hingewiesen.

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## Studies on the Regulation of Respiration in Heavy Work.

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In previous investigations (NIELSEN 1936) it was shown that the increase in pulmonary ventilation in exercise is not caused by an increase in  $\text{CO}_2$ -tension or in acidity of the blood, but is due to an augmentation of the excitability (lowering of the threshold value) of the respiratory centre towards  $\text{CO}_2$ . It was shown, further (ASMUSSEN, CHRISTENSEN and NIELSEN 1943), that the increase in excitability of the centre during light work is not of humoral but of nervous origin. In other experiments (ASMUSSEN, NIELSEN and WIETH-PEDERSEN 1943) it was demonstrated that the nervous influence on the centre was not due to cortical impulses, and it was concluded that the increase in excitability of the respiratory centre was caused by reflexes arising in the exercising parts of the body. This conclusion is supported by experiments of HARRISON, CALHOUN and HARRISON (1932) who showed on dogs that passive movements of the hindlegs, which were only in nervous connection with the rest of the body, produced a small increase in ventilation. COMROE and SCHMIDT (1943) have repeated and extended the experiments of HARRISON et al. and have also reached the conclusion that proprioceptive impulses from the moving limbs play a part in the increase of pulmonary ventilation during muscular work.

In the above mentioned experiments in which the increased excitability of the respiratory centre during muscular work was analyzed, the work was carried out only at small intensities.



In heavy work it has been shown (NIELSEN and HANSEN 1937), that the ventilation can be lowered very considerably by breathing air enriched with oxygen whereas oxygen breathing has no such effect on the ventilation at small work intensities. Since it is unlikely, that oxygen breathing should affect the proprioceptive reflexes in heavy work, but not in light work, it seems justifiable to assume that other factors are involved in the regulation of respiration in heavy work.

It is characteristic for heavy work that the oxygen supply to the working muscles begins to become inadequate. It might therefore be supposed that substances liberated in muscles working under partly anaerobic conditions act on the respiration and that the above mentioned effect of oxygen breathing on the ventilation is due to a more adequate oxygen supply diminishing the production of anaerobic metabolites.

In the present paper this problem has been submitted to a closer examination. Different kinds of work in which a beginning inadequacy of the oxygen supply is to be expected, *i. e.* heavy work with large muscle groups (bicycling), work with small muscle groups (work with the arms) and work in light CO-poisoning, have been studied. The blood lactate has been used as an indicator of the degree of the anaerobiosis.

### Methods.

The work was carried out on a Krogh bicycle ergometer, which for work with the arms could be modified in such a way, that the subject sitting on a high stool could turn it by means of a couple of handles replacing the pedals (compare CHRISTENSEN 1931 and NIELSEN 1938). The pulmonary ventilation and the respiratory metabolism were determined by the Douglas-bag method. The respiratory frequency was registered on a drum and the alveolar gas tensions calculated by means of Bohr's formula.

The blood lactate was determined according to EDWARDS (1938) on capillary blood from a finger or, during work with the arms, from the ear lobe. Determinations of arterial oxygen saturation were made on arterialized capillary blood: The hand was hyperaemized by means of hot water (46—47° C.) for 5 minutes and the blood samples were taken into 0.5 cc syringes from small celluloid tubes fixed to the finger tip as described by MOOK (1931) and BOJE (1934). The dead space of the syringes was filled with

a solution of heparin in 0.9 % NaCl and the syringes calibrated to deliver a known volume (about 0.5 cc) of blood. The blood was transferred directly from the syringes into the cup of the Van Slyke apparatus and analyzed for oxygen content. The oxygen capacity of the blood was determined on another blood sample that had been rotated for about 15 minutes in air.

In experiments, in which pure oxygen or gas mixtures different from air were breathed, the inspiratory side of the valve was connected with a Douglas bag that could be properly filled from cylinders containing the gases.

As main subject served a young well trained man, and the experiments were all carried out in the morning under standard conditions.

### Results.

Fig. 1 shows the pulmonary ventilation ( $V_{37}$ ; 37° C., prevailing barometric pressure and saturated) during work with the legs (bicycling) in relation to the oxygen consumption. The upper curve represents the air breathing experiments and the lower curve the  $O_2$  breathing experiments. In the latter experiments the oxygen uptake was not determined, but was assumed to be equal to the oxygen uptake at corresponding work intensities while breathing air. This assumption is correct for light and moderate work, but in heavy work the oxygen uptake is somewhat larger. However, at work intensities in which a steady state can be reached this difference is small only (compare NIELSEN and HANSEN 1937). The curve representing the experiments in air shows an approximately linear relationship between  $V_{37}$  and oxygen consumption up to about 2.2 l  $O_2$ /min. At higher oxygen consumptions the curve shows a relatively larger increase of  $V_{37}$  as related to the  $O_2$ -uptake. At the small work intensities the ventilations in the oxygen experiments are almost the same as in the air experiments or showing a tendency to be a little higher. But at oxygen consumptions above 1.1 l/min. the ventilations in the oxygen experiments become lower than in the air experiments and the difference is increasing, absolutely and relatively, up to the maximal oxygen consumption of 3.6 l/min, where the difference is 30 l/min or 31 pCt. of the ventilation while breathing air. At the highest work intensities (oxygen consumptions from 3 l/min. and upwards) also the curve representing the oxygen experiments rises more steeply than at the lower work intensities.

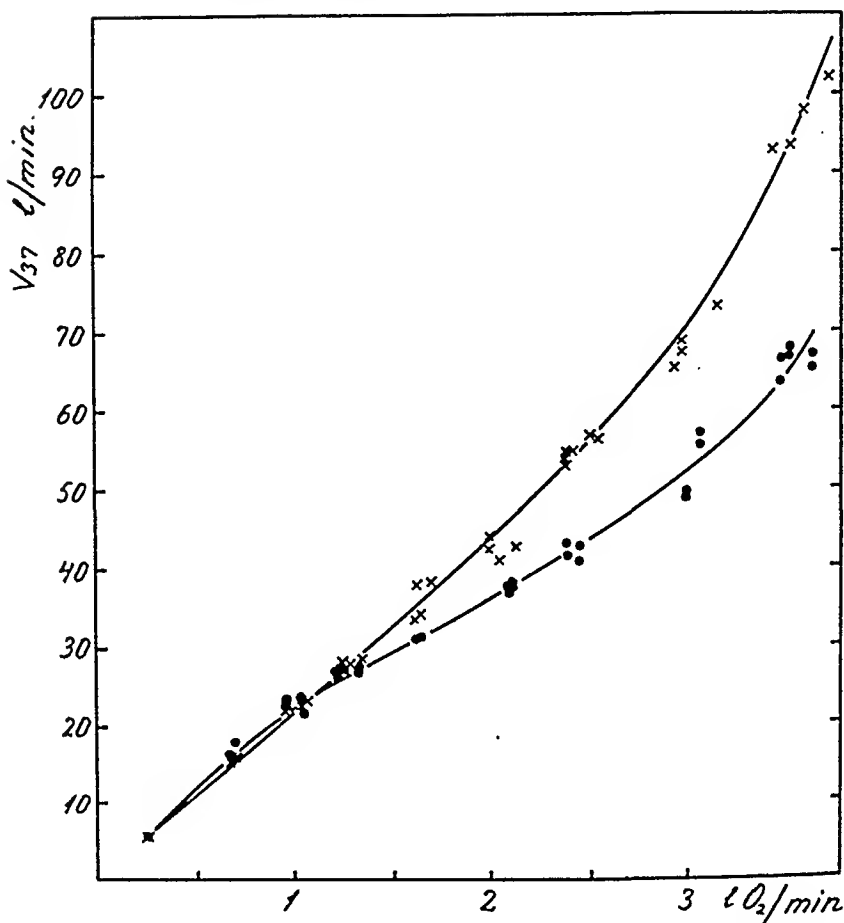


Fig. 1. Ventilation ( $37^\circ \text{C.}$ , prevailing bar. pressure, saturat.) in relation to oxygen uptake. Work with the legs.

× — × in air.

● — ● in 100 pCt. oxygen.

Fig. 2 shows the ventilation,  $V_{37}$ , from the experiments in which work was performed with the arms, plotted against the oxygen consumptions. For comparison the curves from fig. 1 are here redrawn as dashed lines. It will be seen that the curve representing the experiments in air lies for the whole range studied above the curves representing the experiments in which work was performed with the legs. A relatively high lung ventilation for arm-work compared with leg-work has been demonstrated earlier by CHRISTENSEN (1932). Further it can be seen that above a certain work

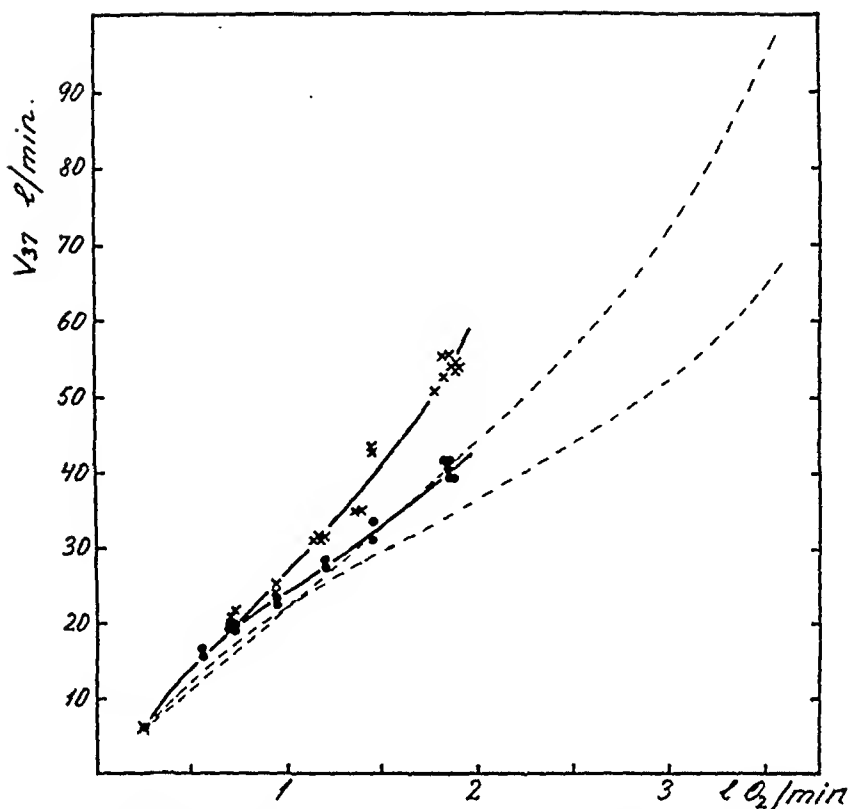


Fig. 2. Ventilation (37° C., prevailing bar. pressure, saturat.) in relation to oxygen uptake.  
 Full lines: work with the arms.  
 Dashed lines: work with the legs (experiments from fig. 1).  
 × — × in air.  
 ● — ● in 100 pCt. oxygen.

intensity oxygen breathing also during work with the arms diminishes the ventilation. This effect of the oxygen breathing is at all oxygen consumptions, both absolutely and relatively, larger than during work with the legs.

In fig. 3 are presented the alveolar  $CO_2$  tensions corresponding to the experiments in fig. 1 and fig. 2. It can be seen that the  $CO_2$ -tensions from the experiments in which work was performed with the legs while breathing air is constant or slightly increasing up to an oxygen consumption of about 2 l/min. At higher oxygen consumptions it decreases steadily to values well beneath the resting  $pCO_2$ . In the corresponding oxygen experiments the alveolar  $pCO_2$  is during rest and at small work intensities a little

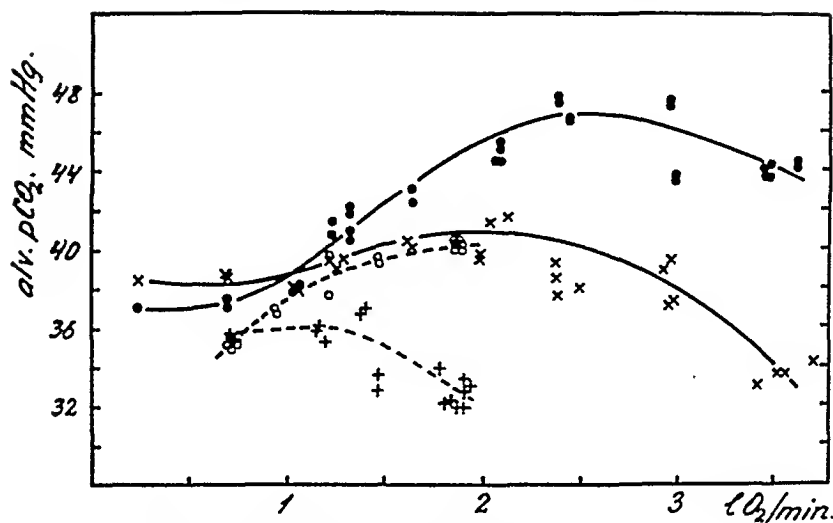


Fig. 3. Alveolar  $\text{CO}_2$ -tensions at various oxygen uptakes.  
Full lines: work with the legs.  $\times - \times$  in air,  $\bullet - \bullet$  in 100 pCt. oxygen.  
Dashed lines: work with the arms.  $+ - +$  in air,  $\circ - \circ$  in 100 pCt. oxygen.

lower than in the air experiments. From oxygen consumptions of about 1 l/min. it becomes higher than in the corresponding air experiments and is steadily increasing up to a value of about 47 mm Hg (at an oxygen consumption of about 2.5 l/min.) after which a slight decrease sets in. When work is performed with the arms the  $\text{pCO}_2$  is considerably lower than in the leg-work experiments, and similar differences between the air experiments and the oxygen experiments are found in these experiments.

In fig. 4 the blood lactates from the experiments in fig. 1 and 2 are plotted against the oxygen consumption. The blood samples were taken after 10, resp. 17 minutes of work, *i. e.* just before and just after the determination of the ventilation was made. The points in fig. 4 represent the averages of these two values. In the leg work experiments in air the blood lactate shows a steep increase from oxygen consumptions of about 2 l/min., but a comparison between the oxygen and the air experiments shows that the two curves separate already at an oxygen consumption of about 1.2 l/min., *i. e.* at the same value at which the ventilation curves (fig. 1) are separating. With increasing oxygen consumptions the difference between the blood lactate concentration in the air experiments and the oxygen experiments increases also. At an

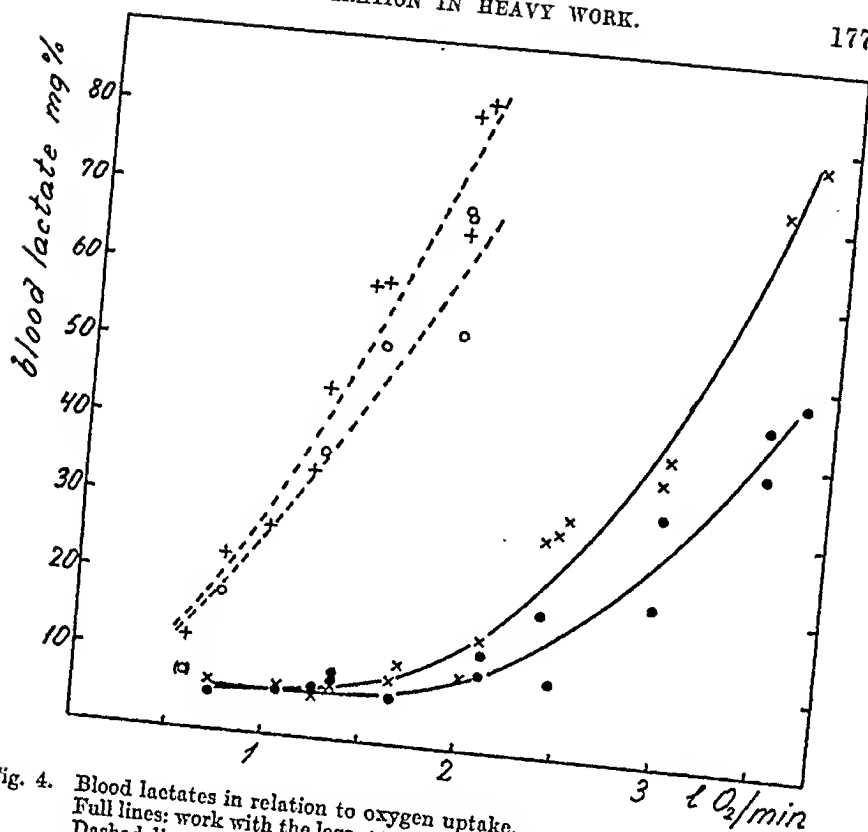


Fig. 4. Blood lactates in relation to oxygen uptake. Full lines: work with the legs.  $\times$ — $\times$  in air,  $\bullet$ — $\bullet$  in 100 pCt. oxygen. Dashed lines: work with the arms.  $+$ — $+$  in air,  $\circ$ — $\circ$  in 100 pCt. oxygen.

oxygen consumption of 3.6 l/min. it is about 32 mg% or 40 pCt. of the value from the air experiments. The blood lactates from the arm-work experiments show a much steeper increase than during work with the legs, and here also oxygen breathing seems to diminish the lactate formation, but the curves for the arm-work determinations are not so well defined as the corresponding curves for the leg experiments, probably owing to the difficulties in taking the blood samples from the earlobes during the arm work.

Fig. 5 shows blood lactate determinations and ventilations ( $V_{37}$ ) from work experiments of 1,440 mkg/min. performed with the legs, while breathing air, 30 pCt., 60 pCt. and 100 pCt. oxygen. It is evident that with increasing oxygen content of the inspired air there is a considerable and gradual lowering of the blood lactate curves and a corresponding decrease in ventilation.

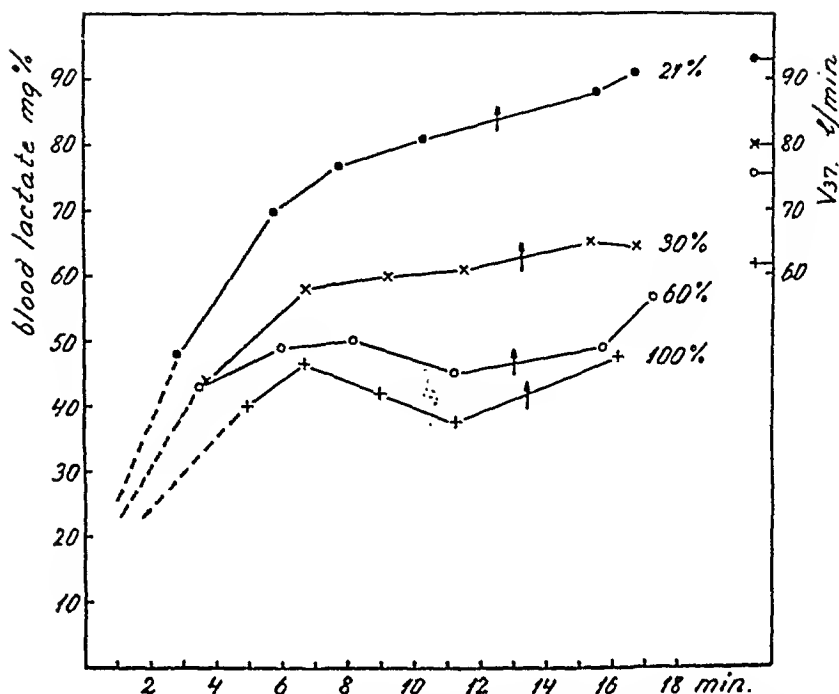


Fig. 5. Blood lactate concentration during work with the legs (1,440 mkg/min.) while breathing: ● — ● air, × — × 30 pCt. oxygen, ○ — ○ 60 pCt. oxygen and + — + 100 pCt. oxygen.

At the right ordinate are shown the corresponding ventilations ( $V_{37}$ ) determined at the points of time indicated by the arrows.

Table 1 shows ventilations ( $V_{37}$ ) and blood lactates from work experiments in which the oxygen supply was hampered by CO-poisoning (about 30 pCt. HbCO). The figures from the treadmill experiments are taken from experiments which were carried out for other purposes by ASMUSSEN and CHIODI (1941). It can be seen, that the two subjects act differently. In subject E. A. there

Table 1.

Subj.	Work	Condition	O <sub>2</sub> -uptake l/min.	V <sub>37</sub> l/min.	V <sub>37</sub> /10 <sub>2</sub>	Alv. pCO <sub>2</sub> mm Hg	Blood lactate mg%
E. A.	Walking on treadmill	Normal	1.81	38.6	21.3	45	13
		CO-pois.	1.76	39.4	22.4	—	24
"	Bicycling 720 mkg/min.	Normal	1.49	29.6	19.9	—	14
		CO-pois.	1.64	37.1	22.6	—	43
H. C.	Walking on treadmill	Normal	1.85	41.9	22.6	44	21
		CO-pois.	2.00	62.0	31.0	32	81

is only a small increase in the blood lactate in the CO-poisoned condition and a corresponding small increase in the ventilation and in the ventilation per l oxygen uptake. In the less well-trained subject H. C. there is a large increase in the blood lactate during CO-poisoning and, corresponding to this, the ventilation per l oxygen uptake is considerably increased and the alveolar  $p\text{CO}_2$  diminished.

### Discussion.

The results presented in this paper show that during work in which the blood lactate concentration is increased *e. g.* heavy work with the legs, work with the arms and work in light CO-poisoning, the pulmonary ventilation is relatively high. It is further shown that oxygen breathing diminishes the blood lactate concentration and at the same time causes a decrease in the ventilation. This effect on blood lactate and the ventilation is gradually increasing with increasing oxygen percentage of the inspired air (fig. 5). Also the CO-experiments (table 1) show a relatively much increased ventilation in the cases where the blood lactate concentration was considerably increased, whereas the CO-poisoning had only a slight effect on the ventilation when the increase in blood lactate concentration was small. All these results suggest a close relationship between the degree of anaerobiosis in the working muscles, as indicated by the blood lactate concentration, and a relatively high ventilation. It is, therefore, natural to assume that some substance produced in the working muscles under partly anaerobic conditions increases the ventilation and that the effect of oxygen breathing on the ventilation is due to a reduced formation of such substances.

It is, however, necessary to investigate whether the above mentioned effect of oxygen breathing on the ventilation can be explained in other ways. It is well known, that a diminished arterial oxygen tension stimulates the ventilation through its action on the chemoreceptors of the aortic and carotid bodies. If the arterial oxygen saturation in heavy work were lowered the effect of oxygen breathing might therefore be explained as due to a diminished reflex activity of these chemoreceptors. In a series of experiments the arterial oxygen saturation was therefore determined in rest and during the heaviest work (1,440 mkg/min.). In 12 rest experiments it was found to be on an average  $97.5 \pm$



0.6 pCt. and in 22 work experiments  $95.7 \pm 0.7$  pCt. The small difference between the results from the two series of experiments is probably trustworthy, but an arterial oxygen saturation as high as 95.7 pCt. can not be assumed to increase the reflex activity of the chemoreceptors to any significant degree (compare EULER, LILJESTRAND and ZOTTERMAN 1939). From this it is concluded that the effect of oxygen breathing on the ventilation during heavy work is not due to an elimination of a pre-existing oxygen lack in the arterial blood.

During heavy work oxygen breathing diminishes the lactic acid formation very considerably, and it might be thought that a decreased acidity of the blood were the reason for the lowering of the ventilation during oxygen breathing. It has been shown, however (NIELSEN 1936), that during oxygen breathing (45 pCt.) the ventilation in heavy work is decreased to such a degree that the acidity of the arterial blood, because of the increase in the alveolar  $p\text{CO}_2$ , is actually higher during oxygen breathing than during breathing of normal air. The effect of oxygen breathing, therefore, can not be due to a decrease in acidity of the arterial blood. Nor is it—as will be shown later (page 185)—due to the decrease in the concentration of the lactic acid itself.

Also in the present experiments the alveolar  $p\text{CO}_2$  in heavy work was found to be much higher during oxygen breathing than during breathing of ordinary air (fig. 3). It might therefore be thought that oxygen breathing in heavy work causes a diminishing of the excitability of the respiratory centre towards  $\text{CO}_2$ . But in that case it seems peculiar that oxygen breathing during rest and during light work should apparently have the opposite effect on the excitability of the respiratory centre as indicated by the position of the curves in fig. 1 and fig. 3. In order to study more closely the effect of oxygen breathing on the respiratory centre at low and high ventilations two series of experiments were performed in which the ventilation in rest was increased by means of  $\text{CO}_2$  breathing. In one series the  $\text{CO}_2$  mixtures consisted of  $\text{CO}_2$  and ordinary air and in the other series of  $\text{CO}_2$  and oxygen. The  $\text{CO}_2$ -breathing experiments were carried out by the same technique as described by NIELSEN (1936) and special care was taken to insure that the determinations were not made before an equilibrium between the alveolar  $p\text{CO}_2$  and the ventilation had been reached, *i. e.* after about 45 minutes at the high  $\text{CO}_2$  percentages.

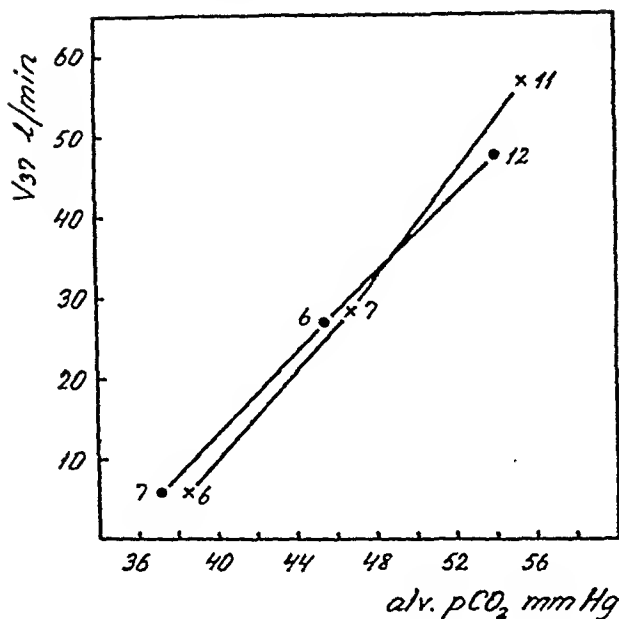


Fig. 6. The ventilation in rest at various alveolar  $\text{CO}_2$ -tensions.  
 × — × breathing mixtures of  $\text{CO}_2$  and air.  
 • — • breathing mixtures of  $\text{CO}_2$  and oxygen.  
 The figures indicate number of single determinations.

The average values from these experiments are presented in fig. 6. It can be seen that the pulmonary ventilation at the high alveolar  $\text{CO}_2$ -tensions is somewhat lower in the oxygen experiments than in the air experiments. This effect of high oxygen on the ventilation at increased  $\text{CO}_2$ -tension has formerly been demonstrated by LINDHARD (1911). At the starting values (no  $\text{CO}_2$  in the inspired air) and at small increases in the  $\text{CO}_2$  tensions the oxygen has the opposite effect, *i. e.* the ventilation is a little higher in the oxygen experiments than in the air experiments. Also in LINDHARDS experiments there is a small decrease in the alveolar  $p\text{CO}_2$  and a slight increase in the alveolar ventilation at the starting values when breathing oxygen. A small increase in the ventilation during oxygen breathing has also been found by other authors (compare review by BEAN 1945). The effect of the oxygen in the  $\text{CO}_2$ -experiments is to some extent similar to the effect of oxygen during the work experiments (fig. 1 and fig. 3). At the high ventilations oxygen breathing causes in both cases a lowering of the ventilation and an increase in the alveolar  $p\text{CO}_2$ , but the effect is much larger during work than during  $\text{CO}_2$ -

breathing. In rest and at low ventilations oxygen breathing in both cases causes a small increase in ventilation and a lowering of the alveolar  $\text{CO}_2$ -tension.

The intersecting curves in the  $\text{CO}_2$ -experiments (fig. 6) can be explained in the way that a high oxygen tension acts on the respiration in two different ways, viz. directly on the respiratory centre and reflexly through the chemoreceptors. It is well known that low oxygen tensions increase the ventilation through a stimulation of the chemoreceptors, whereas it has a depressing effect on the respiratory centre itself, as shown by the fact that breathing of low oxygen decreases the ventilation, when the chemoreceptors have been denervated. The effect of high oxygen tensions on the chemoreceptors is a depressing one (EULER, LILJESTRAND and ZOTTERMAN, 1939) whereas the effect on the respiratory centre itself is to increase its activity, as indicated by the experiments of WATT, DUMKE and COMROE (1943), who showed that oxygen inhalation increases the ventilation when the chemoreceptors had been denervated. Since in normal man oxygen breathing in rest and in conditions with moderately elevated respiration (fig. 6) increases the ventilation and diminishes the alveolar  $\text{pCO}_2$  it must be assumed that the activating effect of the high oxygen tension on the centre here overbalances its depressing effect on the chemoreceptors (compare BERNTHAL 1944). The fact that oxygen breathing at the high  $\text{CO}_2$ -tensions (fig. 6) lowers the ventilation can be explained in the way, that the depressing effect of the high oxygen tension on the chemoreceptors here overbalances the activating effect on the centre. In the normal resting condition the activity of the chemoreceptors is only slight, so that a depressing effect of high oxygen tensions is possible only to a very limited degree. With increasing  $\text{CO}_2$ -tensions the activity of the chemoreceptors is increasing (EULER, LILJESTRAND and ZOTTERMAN 1938 a. o.) so that the depressing effect of high oxygen tension may be able to have a more pronounced effect on the ventilation. This explanation is only possible if one assumes that high oxygen tensions and high  $\text{CO}_2$ -tensions act on the same elements in the chemoreceptors. That this is the case is supported by the observations of EULER, LILJESTRAND and ZOTTERMAN (1939), that oxygen want and  $\text{CO}_2$  surplus act on identical receptors.

The effect of oxygen breathing on the ventilation during work can be explained in a similar way as during  $\text{CO}_2$ -breathing. The

tendency to an increase in ventilation and the lowering of the alveolar  $p\text{CO}_2$  during light work (fig. 1 and 3) can be explained by assuming that the activating effect of the high oxygen tension on the respiratory centre overbalances the depressing effect on the chemoreceptors. The large decrease in ventilation caused by oxygen breathing in heavy work can, according to the above, be explained by the assumption, that oxygen breathing during heavy work has two distinct effects, namely 1. to diminish the anaerobic production of substances which increase the ventilation as mentioned on page 179 and 2. to depress the effect of such substances on the chemoreceptors — just as it was assumed that oxygen breathing depresses the chemoreceptor activity, when this is increased by high  $\text{CO}_2$ -tension.

This assumption also offers an explanation to the finding that oxygen breathing has a relatively greater effect on the ventilation in work performed with the arms than in work with the legs (fig. 2) in spite of the fact that in the former case the effect on the anaerobiosis — as indicated by the concentration of the blood lactates (fig. 4) — seems to be the smaller: The higher blood lactate concentration during arm work suggests a higher chemoreceptor activity, so that the depressing effect of oxygen breathing can be more pronounced.<sup>1</sup>

From the present experiments it is concluded that during heavy work some substance which increases the ventilation via the arterial chemoreceptors is produced in the working muscles. The occurrence of this substance seems to be rather closely correlated to the concentration of lactic acid in the blood. It seemed possible, therefore, that the substance might be lactic acid itself. This possibility has been studied in experiments, the results of which are presented in fig. 7.

In experiment I the inspired air was normal atmospheric air from the beginning of the experiment until a steady state of ventilation had been reached, when the inspired air was suddenly changed from air to pure oxygen. After five minutes of oxygen breathing (16 min. of work) the inspired air was again suddenly changed to ordinary air. In experiment II the inspired air was pure oxygen from the beginning, and the change to ordinary air

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<sup>1</sup> When the ventilation in light arm-work, where oxygen-breathing apparently has no effect, is somewhat higher than during work with the legs, it might be due to differences in the proprioceptive reflexes from the limbs in the two kinds of work.

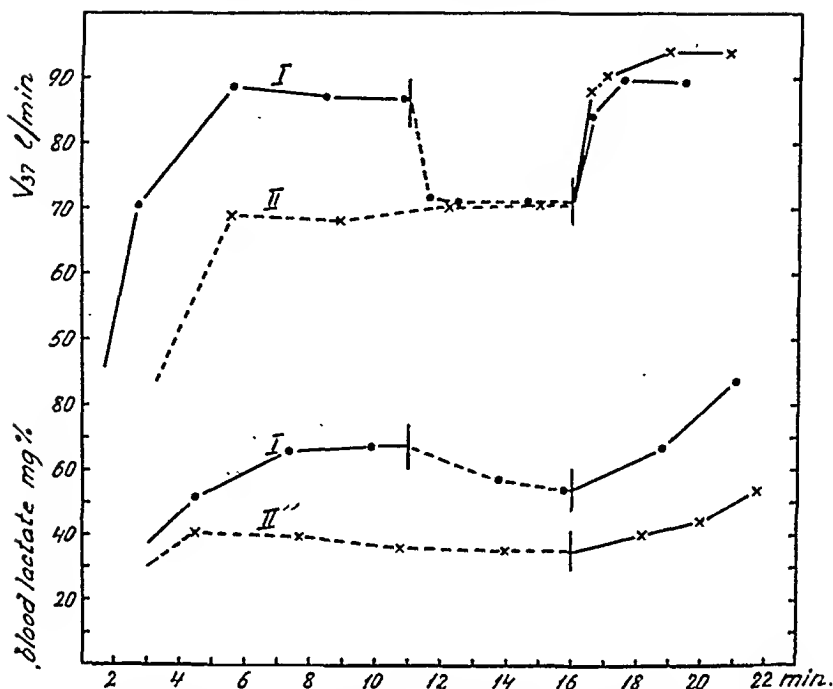


Fig. 7. The ventilation ( $V_{37}$ ) and the blood lactate concentration during work with the legs (1,440 mkg/min.). Experiment I (● — ●): start while breathing air (full lines); after 11 min. sudden change to 100 pCt. oxygen (dashed lines) and after 16 min. back to air breathing (full lines). Experiment II (× — ×): start while breathing 100 pCt. oxygen (dashed lines); after 16 min. change to air (full lines).

was made at the same point of time in the experimental period (16 min. of work) at which the oxygen breathing in experiment I was again changed to ordinary air.

The experiments show that the ventilation in experiment I at the change to oxygen breathing suddenly decreases to practically the same value as in experiment II whereas the lactate concentration is only slightly affected during the five minutes of oxygen breathing. The sudden decrease in ventilation can be explained as the result of the depressing effect of the high oxygen tension on the chemoreceptors and a rapid decrease in concentration of the active substance. At the change from oxygen breathing to air breathing the ventilation in both experiments increases at first rapidly and then approaches values slightly above the values in experiment I before the change to oxygen breathing was made.

This change in ventilation can be explained as being due to the sudden disappearance of the depressing effect of the high oxygen tension, and to a rapid increase in the concentration of the active substance to values corresponding to air breathing conditions.

The blood lactate is during the air breathing (after 16 min. of work) considerably higher in experiment I than in experiment II (up to about 35 mg%), and, if anything, the ventilation is the highest in the experiment in which the lactate concentration is the lowest. From this it is concluded that the active substance which increases the ventilation is not lactic acid.

In the present investigation an identification of the active substance has not been attempted, but it can be said that the substance is produced in increasing amounts by increasing anaerobiosis in the muscles, and that it has properties allowing it to appear and disappear much more rapidly from the blood stream than the lactic acid.

From previous investigations the increase in pulmonary ventilation during light work (see the introduction) is assumed to be due to an increased excitability (lowered threshold value) of the respiratory centre towards  $\text{CO}_2$  caused by reflex impulses from the limbs. The oxygen experiments in fig. 3 (work with the legs) seem to show that the reflex impulses in this subject are responsible for the increase in ventilation only up to work intensities corresponding to oxygen consumptions of about 1 l/min. At higher work intensities the alveolar  $\text{pCO}_2$  in the oxygen experiments is increasing up to work intensities corresponding to oxygen consumptions of about 2.5 l/min. The increase (about 9 mm) is so large that a corresponding increase in the alveolar  $\text{pCO}_2$  during  $\text{CO}_2$ -breathing in rest would increase the ventilation 23 l/min. (compare fig. 6) *i. e.* the same increase in ventilation as occurs when the oxygen consumption during work (fig. 1, oxygen breathing) is increased from 1 l/min. to 2.5 l/min. Consequently in this range of work intensities *the increase* in pulmonary ventilation in this subject while breathing oxygen can be explained in the classical way as due to an equilibrium being established between an increased alveolar  $\text{pCO}_2$  and an increased ventilation, so that in this range the excitability (threshold value) of the centre remains constant. At oxygen consumptions higher than 2.5 l/min. the alveolar  $\text{pCO}_2$  is again lowered, presumably because the concentration of the active substance discussed in this paper becomes so high that in spite of the depressing effect of the high

oxygen tension (compare page 183) it now stimulates the chemoreceptors and causes the further increase in ventilation.

In the air breathing experiments the alveolar  $p\text{CO}_2$  in rest and during work is nearly constant up to very considerable work intensities. As there is no reason to believe that the reflexes from the working limbs during normal conditions and during oxygen breathing are different and as there is no essential increase in the normal chemical stimulus for the respiratory centre (arterial  $p\text{CO}_2$ ) it is likely that the further increase in ventilation — corresponding to oxygen consumptions of about 1 l/min. and upwards — is due mainly to an increasing concentration of the active substance produced under partly anaerobic conditions (compare fig. 1 and fig. 4).

### Summary.

The regulation of the respiration has been studied in different kinds of muscular exercise, in which the muscles are working under partly anaerobic conditions, viz.: heavy work with the legs, work with the arms and work in light CO-poisoning.

It is shown that a close relationship between the degree of anaerobiosis — as indicated by the concentration of the blood lactates — and a relatively high lung ventilation exists.

It is further shown that when the muscles are working under partly anaerobic conditions (increased blood lactate concentration) oxygen breathing lowers the concentration of the blood lactates, and the ventilation, and that the lowering is proportional to the percentage of the oxygen in the inspired air. In rest and during light work (no increased blood lactate concentration) the breathing of oxygen on the other hand tends to produce a slight increase in ventilation and a decrease in alveolar  $p\text{CO}_2$ .

The effect of oxygen breathing on the ventilation during partly anaerobic work is not due to a decrease in acidity (see page 180) of the arterial blood or to the elimination of a previously existing arterial unsaturation.

It is made probable, that the different effect of oxygen breathing in light and heavy work is due to the circumstance that oxygen breathing acts on the respiration in two different ways, viz. activating on the respiratory centre itself, and depressing on the activity of the chemoreceptors. It is assumed that in rest and during light work in which the activity of the chemoreceptors is

kreises (TONNER), wo die Flächengrösse und die Intensität als gesonderte Phänomenalitäten erlebt werden können.

Bei grösseren Flächen gelten Regeln von komplizierterer Form, wie dies besonders WALD klar und theoretisch begründend, gezeigt hat. Unser Fall, die »punktuelle« Reizung, gehört aber nicht hierher.

Die beiden Einschränkungsrelationen, die zeitliche und die flächengemässe können beide als *Minimalregeln* bezeichnet werden, eben wegen ihrer Gültigkeit nur innerhalb bestimmter phänomenal bedingter Grenzen. Die Regeln oder Ausdrücke betreffen begriffliche physikalische Grössen, beziehen sich aber auf phänomenale Gleichheit: Reizintensität mal Reizzeitdauer (Reizfläche) ist gleich einer Konstante, *wenn* es sich um phänomenal gleiche (und momentane bzw. punktuelle) Erlebnisse handelt. Auch die Grenzen der Gültigkeit der Regeln sind phänomenal bestimmt: die Momentanität bzw. die Punktualität (Empfindungsfläche) des Erlebens. Eine Behandlung der Bedeutung dieser Regeln dürfte nicht möglich sein ohne auf das Problem des Verhältnisses vom Phänomenalen und Reizbegrifflichen einzugehen. Dies gehört aber nicht zum Thema dieser Abhandlung, soll aber in einer späteren Arbeit aufgenommen werden (REENPÄÄ, 1947).

Die beiden Minimalregeln können schliesslich zusammengefasst werden, zu einer allgemeineren Einschränkungsrelation  $L \cdot f \cdot t = \text{Konstante}$ , welche besagt, dass die das Erlebnis begrifflich wiedergebenden Reizgrössen der Lichtintensität, der Flächengrösse und der Zeit einander gemäss der Produktenregel einschränken, wenn es sich um phänomenal in allen Beziehungen minimale Erlebnisse handelt (momentane und punktuelle Erlebnisse). Bei grösseren Flächen oder Zeiten, die phänomenal flächenhaft wirken bzw. eine phänomenale Dauer besitzen, kann von einer entsprechenden Einschränkungsrelation nicht gut gesprochen werden, auch wenn gewisse empirische Exponentialformeln die Abhängigkeit der Reizbestimmungsstücke auch hierbei interpolatorisch auszudrücken vermögen. Die momentan-punktuelle Minimalregel besagt, nach unserem Dafürhalten, mehr als die obenerwähnten Regeln; sie dürfte etwas von dem Zusammenhang des Phänomenalen und der Begriffsgrössen besagen, wenn es sich um schwellenmässige, differentielle Phänomenalitäten handelt. Die schwellenmässigen phänomenalen Erlebnisse oder Beobachtungen ergeben die einfachen Regeln, den *bilinearen* bzw. *trilinearen* Ausdruck betreffs der begrifflichen Reizgrössen (wie z. B. unsere



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## Further Observations on Dietary Gizzard Ulcer in Chicks.

By

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It is known that the development of dietary gizzard ulcer in chicks can be accelerated by the incorporation of cinchophen in the diet (CHENEY 1938) and also to some extent by omission of dietary vitamin E (DAM and SEGAL 1945). The same symptom can be retarded by other changes of the diet such as lowering of the salt content and, especially, by the addition of hog liver fat. The effect of hog liver fat is associated with the highly unsaturated fraction of the fatty acids (DAM and SEGAL 1945). The present studies furnish some more information on this problem which, although not yet complete, might be of interest to investigators in this field.

The assay technique employed (using cinchophen diets) was the same as in the studies of DAM and SEGAL (1945). The variations of the diet consisted in introducing the methyl esters<sup>1</sup> of highly unsaturated fatty acids of the type characteristic of hog liver fat such as arachidonic acid and the mixture of fatty acids from the phosphatides of beef adrenals. The effect of these acids was compared with that of fatty acid mixtures of the type characteristic of fish oils such as Menhaden oil, and also with the effect of omitting cod liver oil from the diet (using other sources of vitamins A and D) and of increasing the content of cod liver oil.

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<sup>1</sup> This material was generously put at the author's disposal by Dr. J. B. BROWN of The Ohio State University, Columbus, Ohio.

The results are presented in table 1.

Table 1.

Diets	Average Ulcer Score in % of the U. Sc. in the corresponding negative control group	No. of animals autopsied	Duration of Experiment, days	Place of Investigation
1. Basal + 0.5 % highly unsaturated fraction of fatty acids from Menhaden oil . . . . .	89	10	29	Rochester
2. Basal + 1 % of same addition	87	9	29	,
3. Basal + 0.5 % of methyl esters of fatty acids from phosphatides of beef adrenals . . . . .	52	10	29	,
4. Basal + 0.5 % methyl arachidonate . . . . .	64	9	29	,
5. Basal + 5 % hog liver fat . .	50	9	29	,
6. Basal + 0.5 % purified methyl arachidonate . . . . .	54	13	28	{ Rockefeller Inst.
7. Basal with omission of cod liver oil + vitamins A and D as concentrates (2 drops per animal per week) . . . . .	42	10	43	
8. Basal with 10 % cod liver oil instead of the usual 5 % . .	95	12	43	,

According to Dr. BROWN the Menhaden oil used in diets 1 and 2 consisted of the polyethylenic fraction (iodine value 285.2) about 80 to 85 % of which consists of esters of three and more double bonds. Not over 3—5 % of these esters being arachidonate. The preparation from beef adrenals used in diet 3 contained 25—30 % of methyl arachidonate as assayed from the polybromide number method. The methyl arachidonate used in diet 4 was prepared as described by MOWRY, BRODE and BROWN (1942) by distillation-crystallisation procedures. The purified methyl arachidonate used in diet 6 was prepared by reduction of methyl octabromo-arachidate with zinc in neutral methanol. Its iodine value was 321.06, the mol. wt. 318.2.

The experiments show that the development of gizzard ulcers is retarded by the addition of methyl arachidonate or methyl esters of the fatty acid mixture of highly unsaturated fatty acids from the phosphatides of beef adrenals but not so by addition of

similar amounts of the mixture of highly unsaturated fatty acids from Menhaden oil. (Scores of 89 and 87 are not significantly different from 100 which represents the score in the corresponding negative control group.) Omission of cod liver oil from the basal diet also resulted in retardation of the development of ulcers, but increase of the cod liver oil content from 5 % to 10 % did not cause any difference in the tendency to ulceration. Apparently arachidonic and possibly other highly unsaturated fatty acids from adrenal phosphatides act differently from the fatty acids characteristic of fish oil.

Further studies are required to explain the observed effects and to settle the question if and how other fatty acids and fat constituents influence the symptom.

Part of this work was aided by a grant from Wyeth Incorporated of Philadelphia to the University of Rochester. The author expresses his sincere thanks to Dr. J. B. BROWN of The Ohio State University, Department of Physiological Chemistry, for having supplied the methyl esters of the fatty acids used in this study. He also thanks Dr. PAUL GEORGE of the University of Pennsylvania School of Medicine, for having suggested the acceptance of the grant which made it possible for him to work on the present problem in Rochester.

### Summary.

The development of dietary gizzard ulcers in chicks is decreased by the omission of cod liver oil from the diet or by the incorporation of methyl arachidonate or methyl esters of the highly unsaturated fatty acids from phosphatides of beef adrenals in the diet. Incorporation of methyl esters from the highly unsaturated fatty acids from a fish oil such as Menhaden oil increases the tendency to ulceration.

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## Solvent Water in the Human Erythrocytes.

By

S. L. ØRSKOV.

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Several experiments have been made in order to find out how great a part of the erythrocytes acts as a solvent. The problem is theoretically interesting, and it is of importance in connection with investigations on permeability; here it is usual to estimate the solvent water at 50 % of the volume, supported by experiments on the volumes of the erythrocytes at different osmotic pressure, though the watery phase is about 70 %.

The usual way to find the amount of solvent water has been to add an anelectrolyte which can permeate the blood cell membrane, and wait until equilibrium has been reached between the watery phases outside and inside the blood corpuscles and then determine the concentration of the substance in the outer watery phase, and from this calculate the concentration in the inner watery phase. The results do not agree, and it has therefore been found useful to try to elucidate the problem through further experiments. EGE and ROCHE (1930) have determined the glucose concentration in serum and blood corpuscles and found the ratio 100/80, approximately corresponding to the ratio of the water content of serum and blood corpuscles respectively. SOMOGYI (1933) arrives at the same result. He determines the glucose in plasma and blood and calculates the glucose concentration in the blood corpuscles by haematocrit determinations. MAC KAY (1932) finds the glucose concentration in the watery phase of plasma in proportion to the watery phase of the blood corpuscles to vary from 0.97 to 1.03. He assumes the water concentration of the blood corpuscles to be 79.4 % of the plasma concentration. He adds glucose to blood and finds that the concentration is the same in the two watery phases. FOLIN and SVEDBERG (1930)

find a lower glucose concentration in the blood corpuscles than stated in the above mentioned works, whereas they find urea and creatinine in the same concentration in the watery phase of serum and blood corpuscles.

It should be mentioned that glycolysis can diminish the glucose concentration in the red blood corpuscles (SOMOGYI).

PARPART and SHULL (1935) have investigated the solvent water of the erythrocytes of a number of animals. In most of the experiments they first wash the erythrocytes and then add glycol or glycerol; after equilibrium of diffusion has been reached they determine the concentration in the outer fluid. They find that only about 71 % of the watery phase of the erythrocytes serve as a solvent. For the determination they use the same method as WEECH and MICHAELIS (1928), oxidation with potassium dichromate in sulphuric acid and iodometric titration with sodium thiosulphate. In a few experiments they investigate the solvent water of the erythrocytes for urea and arrive at a wholly different result, the concentration of urea in the watery phase of the erythrocytes in 3 experiments is 107, 111 and 104 % of that in the outer phase. They suppose that this is caused by adsorption and refer to HILL (1930) who found a lower vapour pressure of urea and creatinine dissolved in serum and blood than expected. With NaCl, KCl and saccharose he found that 95 to 97 % of serum water act as solvent water. With saccharose and haemolyzed blood cells he finds the same amount of solvent water.

PARPART and SHULL also investigate the volume of the erythrocytes at different osmotic pressures and they find agreement with Boyle-Mariotte's Law if they assume that about 70 % of the watery phase of the erythrocytes acts as a solvent. It was especially their experiments which invited to further experiments.

### Methods.

The blood used was drawn from students and defibrinated. Two samples of 14 ml are pipetted into two 25 ml flasks; to one are added 2 ml 0.9 % NaCl, to the other 2 ml 0.9 % NaCl which are also 1 to 4 m. with regard to the electrolytes employed. The flasks are now placed in a rocking machine until equilibrium of diffusion has been reached, at which time haematocrits of the two samples should be alike.<sup>1</sup> The flasks were rocked for 10 minutes at about 20° in the urea experiments, for 30 minutes in the glycol and glycerol experiments, for 120 minutes in the malonamide experiments, and for 120 minutes

<sup>1</sup> Haematocrits are made in all experiments.

at 37° in the glucose experiments. The haematoerit tubes<sup>1</sup> are centrifuged at 10,000 rounds per minute for 10 minutes after which a constant volume of the erythrocytes has been reached.

Now the rest of the blood is also centrifuged at the same velocity and for the same time. Controls have shown that very little fluid is left between the packed erythrocytes, 1.7—3.2 % of the total volume.

The volume of serum between the centrifuged erythrocytes was determined by adding inulin to blood, centrifuging this, dispersing the erythrocytes in 0.9 % NaCl, centrifuging and determining the concentration of the inulin in the supernatant fluid.

When calculating the results no correction is made on account of the intercellular fluid as it is expected to be the same in all experiments and is of no importance to the results.

The serum is now removed, the last of it carefully with cotton wool, and the blood corpuscles can be pipetted off.

The water content of serum and blood corpuscles was determined in the following way; 1 ml was weighed in a 1 ml pipette, transferred to a weighing glass and the dry matter weighed after 24 hours at 105°.

The solvent water has been determined with 5 anelectrolytes, urea, malonamide, glycerol, glycol and glucose.

For the determinations three methods have been employed.

Determinations were made on serum as well as on the centrifuged blood corpuscles. It is then possible to calculate the percentage of the added substance found in the experiment. No experiment was used where a difference of more than 4 % from the expected was found.

For the urea determinations the method of VAN SLYKE and CULLEN was used.

Two urease (Leo) tablets were used for each sample. In 11 experiments the yield was on an average 100 % of the expected (ranging from 96—104 %).

As to the determination of the other substances the serum and the erythrocytes are first deproteinized.

One ml of serum or erythrocytes is added to 4.6 ml H<sub>2</sub>O, by this the erythrocytes are haemolyzed, further 4 ml 4 % HgCl<sub>2</sub> and 0.4 ml 4 n HCl are added. The next day the mixture is filtered.

The malonamide analysis is rather simple. To a 1 liter retort are added 120 ml H<sub>2</sub>O, 90 ml 33 % KOH, 10 ml 25 % Na<sub>2</sub>S and 15—20 ml filtrate. 200 ml H<sub>2</sub>O and the ammonia are distilled into 45 ml 0.1 n HCl. Titration with 0.1 n NaOH. Filtrate from 1.5—2 ml serum or erythrocytes was used for each analysis. The yield of known amounts was on an average 98.6 % in the experiments 97.4 %.

For this as for all the methods employed it should be emphasized that the titration results from serum and erythrocytes of blood to which only 0.9 % NaCl have been added are used as blanks.

For the determination of glycol, glycerol and glucose the following method has been used.<sup>2</sup>

<sup>1</sup> The tubes used are described in Ørskov (1945).

<sup>2</sup> For glycol, glycerol and glucose it was tried if the deproteinization gives rise to a loss of the substances. For the erythrocytes a very small loss was found (about 2 per cent), for this loss no correction has been made.

In a test tube 5 ml sulphuric acid with silver dichromate (NICLOUX 1927), 3 ml 1 n potassium dichromate and 2 ml of the filtrate are mixed, boiled for half a minute and after 10 minutes transferred to a beaker containing 75 ml  $H_2O$ , 10 ml 10 % KJ and titrated with 0.1 n  $Na_2SO_3$  with starch as indicator. The results show that glycol and glucose are oxidized completely, while glycerol is only partially oxidated but the results with this substance agree just as well. In some experiments the blood was centrifuged and the serum exchanged with the same volume 0.9 % NaCl; after stirring and addition of the anelectrolytes the distribution of these can be examined.

## Results.

The results are given in tables 1 to 6.

At first sight they do not agree very well with previous results.

### Urea.

In all experiments (table 1) the concentration in the watery phase of the erythrocytes is higher than the concentration in the outer watery phase, a little higher with 0.125 m urea in the blood than with 0.5 m and the highest concentration is found with 0.9 % NaCl in the outer solution.

Table 1.

### Urea.

*The urea molarity of the suspension 0.5.*

Blood-spenders	$\frac{\text{Urea conc. of erythr. water}}{\text{Urea conc. of serum water}}$	Average	$\frac{\text{The ratio calculated from urea conc. in serum}}{\text{Urea conc. in serum}}$	Average
Ø .....	1.07		1.15	
H. K. ....	1.04	1.05	1.01	1.10
H. K. ....	1.03		1.14	

### Urea molarity 0.125

Ø. ....	1.06		1.01	
A. J. ....	1.06		1.08	
Ø. ....	1.13	1.08	1.15	1.08
H. K. ....	1.06		1.01	
H. K. ....	1.10		1.13	

	$\frac{\text{Urea conc. of erythr. water}}{\text{Urea conc. of 0.9 \% NaCl}}$	Average	$\frac{\text{The ratio calculated from urea conc. in 0.9 \% NaCl}}{\text{Urea conc. in 0.9 \% NaCl}}$	Average
M. C. ....	1.14		1.04	
M. C. ....	1.09	1.12	1.10	1.07
M. C. ....	1.12		1.07	



PARPART and SHULL found the same with washed erythrocytes. That the highest concentration is found with no proteins in the outer solution is in accordance with HILL, who finds that urea is bound to serum as well as to whole blood. From table 1 can be seen that urea is adsorbed by serum as well as by the erythrocytes and mostly by the latter.

When the concentrations are calculated only from the concentration in the outer solution no difference can be seen caused by different concentration of urea or 0.9 % NaCl but this way of calculation must be considered as decidedly less accurate.

### Malonamide.

With malonamide (table 2) the same concentration is found in the inner and outer watery phase when the erythrocytes are suspended in serum, when suspended in 0.9 % NaCl a somewhat higher concentration is found in the erythrocytes, which might be explained by malonamide being adsorbed in the same degree to serum proteins as to proteins of the erythrocytes.

Table 2.

### Malonamide.

*The malonamide molarity of the suspension 0.125.*

Blood-spenders.	Malonamide conc. of erythr. H <sub>2</sub> O	Average	The ratio calcu- lated from mal- onamide conc. in serum.	Average
	Malonamide conc. of serum H <sub>2</sub> O			
K. P. ....	1.00	1.00	1.03	1.03
K. P. ....	1.00		1.01	
K. P. ....	0.96		1.01	
K. P. ....	1.02		1.08	
	Malonamide conc. of erythr. H <sub>2</sub> O	Average	The ratio calcu- lated from mal- onamide conc. in 0.9 % NaCl	Average
	Malonamide conc. of 0.9 % NaCl			
M. C. ....	1.00	1.05	0.95	1.01
M. C. ....	1.09		1.05	
M. C. ....	1.09		1.05	
M. C. ....	1.02		0.99	

When calculated from the concentration in the outer phase the ratio is reversed though the difference is less.

## Glycol.

Glycol (table 3) shows a little lower concentrations in the watery phase of the erythrocytes when defibrinated blood is used. When serum is exchanged with 0.9 % NaCl the glycol concentration in the erythrocytes is the highest, indicating that glycol is adsorbed in a higher degree to the serum proteins.

Table 3.

## Glycol.

*The glycol molarity of the suspension 0.5.*

Blood-spender	Glycol conc. of erythr. H <sub>2</sub> O	Average	The ratio calculated from glycol conc. in serum	Average
	Glycol conc. of serum H <sub>2</sub> O			
B. O. ....	0.97	0.95	1.03	1.00
H. ....	0.97		0.96	
H. ....	0.93		1.03	
H. ....	0.91		0.98	
	Glycol conc. of erythr. H <sub>2</sub> O	Average	The ratio calculated from glycol conc. in 0.9 % NaCl	Average
	Glycol conc. of 0.9 % NaCl			
B. O. ....	1.08	1.10	1.32	1.20
H. ....	1.09		1.10	
H. ....	1.14		1.17	

Calculated from values in the outer phase the ratios are respectively 1.00 and 1.20.

## Glycerol.

In the experiments with glycerol (table 4) higher concentrations in the erythrocytes are found in all experiments and the exchange of serum with 0.9 % NaCl is of no influence, indicating that glycerol is only bound to the erythrocytes. Calculated from the concentrations in the outer fluid similar conclusions may be drawn though the results are differing in a higher degree.

## Glucose.

Glucose (table 5) behaves as glycerol. Only two experiments with 0.9 % NaCl have been made, and the lower concentration in the erythrocytes than found with defibrinated blood can not be explained.

Table 4.

*Glycerol.**The glycerol molarity of the suspension 0.5.*

Blood-spender	$\frac{\text{Glycerol conc. of erythr. H}_2\text{O}}{\text{Glycerol conc. of serum H}_2\text{O}}$	Average	The ratio calculated from glycerol conc. in serum	Average
B. O. ....	1.04		0.97	
B. O. ....	1.09		1.10	
B. O. ....	1.06	1.06	1.04	1.13
H. ....	1.06		1.19	
H. ....	1.04		1.23	
H. ....	1.04		1.23	

	$\frac{\text{Glycerol conc. of erythr. H}_2\text{O}}{\text{Glycerol conc. of 0.9 \% NaCl}}$	Average	The ratio calculated from glycerol conc. in 0.9 \% NaCl	Average
B. O. ....	1.09		1.11	
B. O. ....	1.05		1.15	
B. O. ....	1.06	1.08	1.24	1.13
H. ....	1.11		1.10	
H. ....	1.10		1.07	

Table 5.

*Glucose.**The glucose molarity of the suspension 0.25.*

Blood-spenders	$\frac{\text{Glucose conc. of erythr. H}_2\text{O}}{\text{Glucose conc. of serum H}_2\text{O}}$	Average	The ratio calculated from glucose conc. in serum	Average
B. O. ....	1.07		1.02	
B. O. ....	1.15		1.08	
B. O. ....	1.07	1.11	1.18	1.11
H. ....	1.07		1.19	
H. ....	1.17		1.10	

	$\frac{\text{Glucose conc. of erythr. H}_2\text{O}}{\text{Glucose conc. of 0.9 \% NaCl}}$	Average	The ratio calculated from glucose conc. of 0.9 \% NaCl	Average
H. ....	1.02	1.04	1.00	1.06
H. ....	1.05		1.12	

Some experiments have been made with Hagedorn and Norman Jensen's method for the determination of blood sugar. The sugar concentration of the blood was increased by 0.385 %. 0.05 cc serum and blood cell mass were used for the analysis. Apart from this the procedure was the same as in the other experiments.

It is seen from the results (table 6) that 10 minutes at 20° is not enough to obtain equilibrium of diffusion. The ratios are somewhat smaller than the ones found at the higher concentration.

Table 6.

*Concentration of the glucose added 0.355 %.*

Method of determination: Hagedorn and Norman Jensen.

Blood-spender	Glucose conc. of erythr. H <sub>2</sub> O		Glucose conc. of serum H <sub>2</sub> O	
	10 min. 20°	Average	60 min. 37°	Average
M. C. ....	102		106	
M. C. ....	102		102	
M. C. ....		102	111	106
M. C. ....	103		102	
M. C. ....	102		108	

### Discussion.

The general conclusion of the experiments is that the employed substances in most of the experiments are found in a higher concentration in the water of erythrocytes than in the outer solution.

Malonamide and glycol are exceptions from this when serum is the outer fluid.

What may be the reason why there is discrepancy with former works?

PARPART and SHULL are the only two, who have arrived at a similar result and only in the case of urea. But in reality EGE and ROCHE and SOMOGYI may be said to have reached similar results in experiments with glucose, because in their reasoning they have assumed that the ratio of water in serum and the erythrocytes is 100/80 whereas I have found it to be 100/76 on an average of 5 persons; BODANSKY (1934) states it at 100/75.8.

If we employ this ratio and EGE's and SOMOGYI's results, we find that there are 5 % more of glucose in the water of the erythrocytes than outside.

MAC KAY has carried out his experiments in almost the same way as adopted in these experiments, adding glucose to blood and determining the reduction in the serum and in the erythrocytes before and after admixture. But he only waits 10 minutes for the diffusion to take place and that is perhaps not enough. In the present experiments (table 5) the haematocrits only became constant after 1 1/2 hour at 37° and the centrifuging began after 2 hours.

It must be admitted that he had a concentration of 0.3—0.5 % glucose in the blood and in these experiments it is 4.5 % and glucose permeates much more slowly at higher concentrations; but otherwise  $Q_{10}$  is 6 for glucose so that the higher temperature will in any case partly compensate for this (BANG and ØRSKOV 1937).

No temperature has been mentioned in MAC KAY's experiments, so it may be assumed they were carried out at room temperature.

With blood of normal glucose concentration one must always remember that glycolysis may lead to a lower concentration of glucose.

In the present experiments with the high glucose concentration glycolysis will probably be of no consequence.

PARPART and SHULL's experiments on glycol and glycerol can not be easily explained. The method used is very similar to the one here adopted.

They wash the blood 4 times in order to remove reducing substances. Perhaps they have not made blanks on the outer solution.

Reducing substances from the blood corpuscles will of course give too high values of glycol and glycerol and thus too low concentrations in the erythrocytes as the concentration of glycol and glycerol in their experiments are calculated from the concentrations in the outer solution.

No doubt more experiments are needed before it has been proved with certainty that anelectrolytes are adsorbed to the proteins (or more improbably to the lipoids) of serum and the erythrocytes and in a varying degree.

The problem of bound water is not yet solved for though this is not found in the case of the examined substances, it might be the case with others. The logical thing to do is to examine the distribution of a permeable ammonia salt. Some preliminary experiments have been made, but they have so far met with difficulties, in particular because some of these salts are noxious to the erythrocytes.

### Summary.

The distribution of 5 anelectrolytes, urea, malonamide, glycol, glycerol and glucose added to defibrinated blood has been examined. In some experiments the serum was exchanged with 0.9 % NaCl.

The methods used are described.

Urea, glycerol and glucose are found in a higher concentration in the watery phase of the erythrocytes than in serum. Glycol is found in a somewhat lower concentration in the erythrocytes than in serum and malonamide in the same concentration in both.

In experiments with 0.9 % NaCl in the outer solution, glycol as well as malonamide are found in higher concentration in the erythrocytes.

It is assumed that the substances can be adsorbed to the colloids of serum and the erythrocytes but in a varying degree.

The discrepancy between previous and the present experiments can partly be explained.

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## The Volume of the Erythrocytes at Different Osmotic Pressure.

### Further Experiments on the Influence of Lead on the Permeability of Cations.

By

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HAMBURGER (1898) was the first to investigate the volume of the erythrocytes at different osmotic pressure. He suspended the erythrocytes in NaCl solutions varying in concentration from 0.6 to 1.5 %. He centrifuged the suspension in haematocrit-tubes invented by himself.

He found that the volume obeyed Boyle-Mariotte's law, when it was assumed that with horse-blood 53.3—56 %, with rabbit-blood 48.7—51 % of the volume of the erythrocytes consisted of water. As the water content is about 70 % of the volume, it means that the volume does not follow the law. That part of the erythrocyte water which does not take part in the volume-changes is usually called "bound" water, and the other part solvent water, though it must be admitted that it has never been proved that "bound" water can not act as a solvent, on the contrary, it has been shown that some anelectrolytes besides being solved in all the water of the erythrocytes are also bound to the erythrocytes (ØRSKOV 1946).

Later authors come to similar results as HAMBURGER, though the amount of solvent water found may differ. EGE (1932) finds that the cell volume at moderate osmotic pressure obeys Boyle-Mariotte's law, but he assumed the dry matter of the cells to be 40 % (see later). GOUGH (1924) and KREVISKY (1930) find the

solvent water to be about 50 %, MACLEOD and PONDER (1933) 64 %, PARPART and SHULL (1935) 71 % of the cell water. PONDER and SASLOW (1930 and 1931) believe that the discrepancy between the osmotic pressure and the volume found is caused by leaking of potassium from the erythrocytes and refer to KERR (1929). But this shows in reality that the erythrocytes even when suspended in diluted serum usually do not lose potassium. They also criticize the haematocrit-method.

DAVSON (1936) repeated the experiments of PONDER and SASLOW and found that the erythrocytes do not become permeable to cations neither in hypotonic nor hypertonic serum (by adding KCl).

He finds that in hypotonic solutions the erythrocytes swell as expected from Boyle-Mariotte's law but in hypertonic solutions they shrink rather less than expected; he does not try to explain the discrepancy, the volume of the cells is found by determining the content of dry matter in the centrifuged cells.

No doubt there are problems unsolved in respect to the volume of the erythrocytes at different osmotic pressure.

It shall here be tried to find sources of error in the determination of the volume of the cells and to find the content of "bound" water in the erythrocytes of different volume.

### Methods.

The haematocrit-determinations play an outstanding part in these investigations. In contrast to PONDER and SASLOW it is found to be a reliable method giving most reproduceable results.<sup>1</sup> After ten minutes centrifuging at 10,000 rounds per minute constant values are reached. Dry matter is determined in 1 ml of centrifuged erythrocytes and serum.

The different osmotic pressures are produced in the following way. To 6 ml defibrinated human blood in a 25 ml flask are added 3 ml of solutions of different osmotic pressure in small drops and at the same time the blood is shaken. After the flask has been shut it is rocked for 10 minutes and the haematocrits made. In this way even addition of  $H_2O$  will cause no or only an insignificant haemolysis.

The solutions are,  $H_2O$ , 0.45 % NaCl, 0.9 % NaCl, 0.9 % NaCl with 0.25 m mannite (or 1.67 % NaCl), 0.9 % NaCl with 0.5 m mannite (or 2.47 % NaCl) and 0.9 % NaCl with 1 m mannite (or 4.13 % NaCl). In table 1 some of these experiments are seen.

The amount of fluid between the centrifuged cells is determined in the following way. To the above mentioned solutions are added inulin,

<sup>1</sup> The tubes used are described in ØRSKOV (1945).



so that they contain from 1—10 % of this substance. 1 ml of centrifuged erythrocytes is suspended in 2 ml of 0.9 % NaCl, centrifuged, 1.5 ml of the supernatant fluid is deproteinized with trichloroacetic acid, and the inulin concentration determined by the method of ALVING, RUBIN and MILLER (1939), correction being made for the change of volume in 0.9 % NaCl. The content of sodium is determined after ashing at 500° using a modified WEINBACH method (1935) used for some years in this laboratory (OTTOSEN 1945).

Potassium is determined by the method of KRAMER and TISDALL (1921). Chlorine is determined after REHBERG (1926), CO<sub>2</sub> after VAN SLYKE and NEILL and total phosphoric acid in serum after precipitation with trichloroacetic acid after LOHMANN and JENDRASSIK (1926).

### Results.

As already mentioned table 1 shows the haematocrit values at different osmotic pressures and further the volume in relation to the volume at isosmotic pressure (0.9 % NaCl being approximately isosmotic) and finally the expected volume after Boyle-Mariotte's law. The dry matter of the erythrocytes in isotonic solutions amounts to 29.2 % of the volume, the expected volume where the dry matter is assumed to be 40 and 45 % of the volume have also been calculated. As seen from the table the deviations at hypoosmotic pressures are rather small, but at hyperosmotic pressures they are quite distinct as also found by DAVSON (1936). If the dry matter of the erythrocytes had amounted to about

Table 1.

Osmotic pressure in proportion to normal	Haematocrits in experiment			Average	Volume % of normal	Haematocrits in experiment			Average	Volume % of normal	Expected volume at diff. volume % dry matter		
	1.	2.	3.			1.	2.	3.			29.2 <sup>1</sup>	40	45
0.62	36.8	36.5	37	36.8	136						142	134	133
0.82	31.5	31.1	31.5	31.4	116						115	113	111
1.00	27.3	27	27.1	27.1	100	27.5	26.8	26.7	27	100	100	100	100
	+ Mannite					+ NaCl							
1.34	23.8	24.2	24.5	24.1	89	23.8	23.1	23.8	23.6	87	82	85	86
1.67	21.3	20.8	21.3	21.1	78	21.3	20.5	20.2	20.7	77	72	76	78
2.39	18.3	17.6	18.1	18	67	18.8	18.1	17.7	18.2	68	59	65	68

<sup>1</sup> Volume % dry matter at isosmotic pressure.

40 % of the volume the calculated volumes at hyperosmotic pressure would have agreed with those found, so that between 78—85 % of the cell water should be solvent.

That the erythrocytes are only slightly damaged by this procedure can be seen from the following 2 experiments (table 2), where haematocrits are made after 10 and 180 minutes rocking of the blood at room temperature.

Table 2.

Osmotic pressure in proportion to normal	Haematocrit values after 10 minutes		Haematocrit values after 180 minutes	
0.62	1.	37	1.	36
	2.	37	2.	36.8
0.82	1.	30.1	1.	30.9
	2.	29.9	2.	30.9
1.00	1.	27.1	1.	27.1
	2.	26.9	2.	26.1
1.34	1.	23.9	1.	24.3
	2.	23.9		
1.67	1.	20.9	1.	20.9
	2.	21	2.	20.9
2.39	1.	18.1	1.	18.0
	2.	18.9	2.	19.0

The volumes are unchanged. The exchange of cations after 10 minutes rocking at different osmotic pressure is negligible as may be seen from table 3.

Table 3.

Osmotic pressure in proportion to normal	mg % K in serum <sup>1</sup> in 4 experiments				mg % Na <sup>1</sup> in the erythrocytes in 2 experiments	
	1.	2.	3.	4.	1.	2.
0.62	14		14	14	36	37
0.82	16		19	16		
1.00	17	16	17	17	32	33
1.67	14	16		14		
2.39	22	14		22	30	32

<sup>1</sup> After 10 minutes at room temperature. The values have been corrected for volume changes.

The interchange of cations which has taken place is so negligible that it does not influence the volumes of the cells.

When the phosphates are determined in serum at different pressure it is found that very little phosphorus leaves the cells.

Is the intercellular fluid influenced by different osmotic pressure? This question has never been closer investigated. The method employed has been described above.

As seen from table 4 the amount of intercellular fluid is diminished in hypotonic solutions and in a less degree augmented in hypertonic solutions.

Table 4.

Osmotic pressure in proportion to normal	Haematocrit Average	Vol. % intercellular fluid in 5 experiments					Average
		1.	2.	3.	4.	5.	
0.62	36	2.5	2.5	2.0			2.3
0.82	29	2.6	2.4	2.6			2.5
1.00	27	3.3	3.2	3.2	3.8	2.2	3.2
2.39	19.9				4.2	2.8	3.5

No doubt the experiments show that even normal erythrocytes make resistance against the packing, and that the presence of crenated erythrocytes means surprisingly little to the packing.

The figures in table 1 have not been corrected for the contents of intercellular water as this would be of little influence on the results. So neither diffusion of salts nor intercellular fluid can explain the discrepancy from Boyle-Mariotte's law.

On reading the work of DAVSON (1936) the thought appeared that the accordance with Boyle-Mariotte's law at hypotonic pressures and not at hypertonic pressures might be caused by the growing content of haemoglobin at higher osmotic pressure. This interpretation is strengthened by the work of McCUTCHEON, LUCKE and HARTLINE (1931) who investigate the volume of the sea-urchin egg by different osmotic pressure. The volume of the eggs follows Boyle-Mariotte's law perfectly, and here the dry matter does not exceed 15 % of the volume.

My results agree fairly well with DAVSON's with the exception that he finds less free water at hypertonic pressure.

ØRSKOV (1935) found that poisoning of the erythrocytes made them permeable to potassium, so that they shrinkled considerably

when suspended in 0.9 % NaCl. Similar experiments have later to a great extent been made by DAVSON (1939, 1940), who has shown that it is usually found that such poisons also make the membrane permeable to sodium and that under some conditions the potassium, under other conditions the sodium, permeates with the greatest velocity.

As this has never been investigated in connection with lead poisoning and one at the same time got erythrocytes with abnormally high haemoglobin content but at isosmotic pressure, it was tempting to carry out a series of experiments with lead poisoning, where the concentrations of sodium, potassium and the other ions were determined.

### Experiments on Lead Poisoning.

First some experiments were made to find a suitable concentration of lead. To 4 ml defibrinated blood were added 2 ml 0.9 % NaCl and varying amounts of 5 % lead acetate. In table 5 the results are seen. Shrinking begins at 5.2 mg % lead in the suspension. This is somewhat more than was found necessary in the above mentioned experiments where rabbit erythrocytes were suspended in serum, this is probably due to the much denser suspension of erythrocytes which is used in these experiments as lead according to experiments of MORTENSEN and KELLOGG (1944) has a greater affinity to the erythrocytes than to serum.

Table 5.

Concentration of lead in mg % . .	0.7	1.8	2.6	5.2	10.4	26	52
Haematocrit after 2 hours at 37° .	28	28	27.6	26	22.7	20	19.3

The following experiments have been carried out with a lead concentration of 45 mg %. To 10 ml blood + 5 ml 0.9 NaCl is added 0.25 ml 5 % lead acetate and the mixture is rocked during the experiment. In fig. 1 is shown the results of 4 experiments of lead poisoning of the blood from the same person.

The average concentrations of potassium in the watery phase of the erythrocytes in relation to the concentrations in the watery phase in serum is found in the curve with the solid circles.

The concentrations of sodium in the watery phase of serum in relation to the concentrations in the watery phase of the erythro-

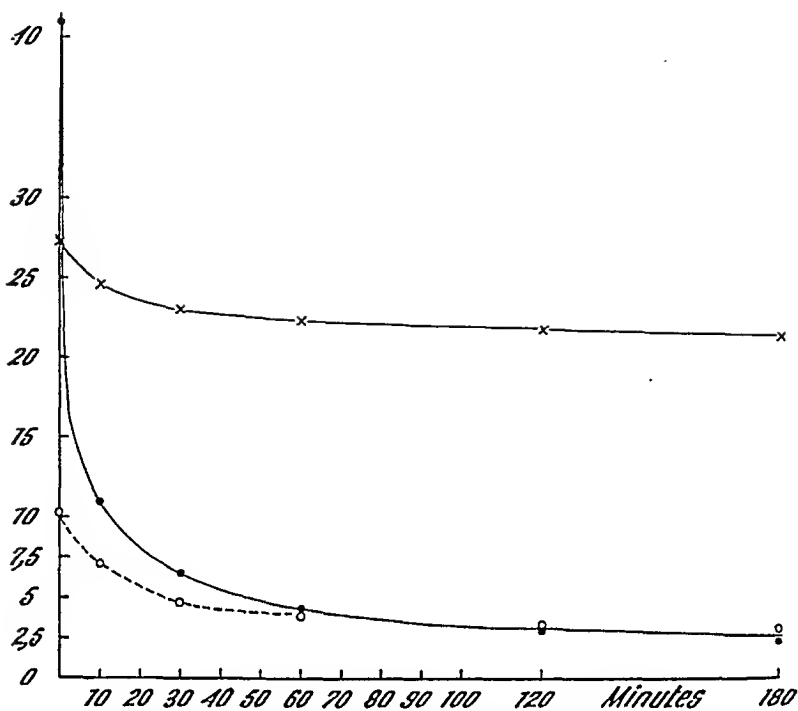


Fig. 1. Abseissa, time in minutes. ordinate  $\frac{K \text{ of cell } H_2O}{K \text{ of serum } H_2O}$  and  $\frac{Na \text{ of serum } H_2O}{Na \text{ of cell } H_2O}$  and haematocrit in vol. % = — x —, K = ● —, Na = ○ —. Lead concentration 45 mg %.

cytes is seen in the curve with the open circles. If this curve is displayed to the right (about 12 min.) until its upper point reaches the first curve it will be seen that the two curves nearly cover each other, it means that sodium and potassium permeate with the same velocity. This result may seem contrary to the former experiments of the author, but only sodium analysis of the blood corpuscles would have revealed the permeability of sodium as well.

In table 6 is demonstrated that the volume of the intercellular fluid after centrifugation is augmented during the experiments and in the same degree as seen in the experiments with different osmotic pressure.

In the above mentioned experiments only potassium and sodium have been determined.

If the osmotic pressure of the watery phase outside and inside

Table 6.

	Min. after addition of lead acetate at 20°					
	Before	10	30	60	120	180
1. Haematocrit . . . . .	34	25	23.8	22	21	20
Vol. % intercell. fluid . . .	1.8	2.1	2.4	2.6	2.6	3.3
2. Haematocrit . . . . .	35	21	21.3	21	21.8	20.8
Vol. % intercell. fluid . . .	1.7	1.8	2.1	2.8	3.6	3.9

Table 7.

	Haematocrit	Serum concentrations (Watery phase)					Cell concentrations (Watery phase)				
		K m. eq.	Na m. eq.	HCO <sub>3</sub> m. eq.	Cl m. eq.	Total m. eq.	K m. eq.	Na m. eq.	HCO <sub>3</sub> m. eq.	Cl m. eq.	Total m. eq.
Normal blood	29.5	2.8	154	13.7	121	291	135	15.6	8.4	82.8	241.8
The same blood + 45 mg % lead	20.6	28.4	128	13.7	122	291.1	69	33	6.9	61	169.9

the erythrocytes should be determined also the anions Cl and HCO<sub>3</sub> had to be analyzed for.

Determinations were made before and 3 hours after addition of lead (room temperature). In table 7 the average results of 4 experiments on the blood of the same person are given. The average concentration per litre of cell water of K + Na + Cl + HCO<sub>3</sub> are 241.8 m. eq. and in serum water 291 m. eq. After addition of lead 169.9 m. eq. are found in cell water and 292 m. eq. in serum water. The difference between serum water and cell water in the controls then is 49.2 m. eq. The corresponding concentrations found by MAIZELS (1936) were 258.5 m. eq. and 291 m. eq. and the difference 32.5 m. eq. The low HCO<sub>3</sub> concentrations are due to no precaution being taken to avoid the escape of CO<sub>2</sub>. The pH in some experiments was found with glass electrode to vary from 7.8 to 8.0.

The excess of plasma osmotic pressure can be explained by assuming that some of the cell water is not "free" in the sense that it is not available for the solution of substances present in

the cell, but it is also possible that the phosphate esters of the cells make up a part of it.

If it is assumed that 49.2 m. eq. are representing osmotic active substances, these will be concentrated when the cells shrink. The haematocrit values in the controls on an average are 29.5, after lead 20.5. The volume of the dry matter of the cell in the controls is 27.5 %.

In the lead poisoned cells 49.2 m. eq. should then be augmented to 84.3 m. eq. When they are added to the 169.9 m. eq. found in the poisoned cells there is still a difference between serum water concentration and cell water concentration amounting to 37.9 m. eq. or 13 % of the serum concentration, when expressed in pressure of atmosphere 0.85.

If it is assumed that the difference between the concentration between serum water and cell water 122 m. eq. is to be explained by the presence of "bound" water, it amounts to 42 % of the serum concentration or 2.7 atmospheres pressure. Comparing these results with those of volume determination at hypertonic pressure, see table 1 and text, and estimating the "bound" water at 15 % the difference in osmotic pressure between serum water and cell water at 2.3 times isosmotic pressure is 93 m. eq. or 2.08 atmospheres.

As the volume of the cells is about the same at this osmotic pressure as after lead poisoning this difference in osmotic pressure is to be compared with the results after blood poisoning.

So the lead poisoning experiments support the conception that by shrinking of the cells the difference of concentration between the serum water and the cell water is increased and it is natural to suppose that the reason is the rising concentration of haemoglobin. But it must be admitted that so many analysis are necessary in obtaining the osmotic pressure of the watery phases that the exactness of the results is somewhat uncertain.

## Discussion.

The experiments show that at hypertonic pressure the erythrocytes do not shrink as much as expected and diffusion of potassium, sodium or phosphates or the amount of intercellular fluid outside the centrifuged cells can not explain the results.

4 possibilities may be considered.

1) Part of the cell water is not available for the solution of substances present in the cells.

2) There is a framework in the cells which tries to resist the shrinking.

3) The haemoglobin molecules are elastic and make resistance against the shrinking of the cells.

4) The salts of the watery phase of the cells exert an augmented osmotic pressure when the haemoglobin concentration is augmented.

Ad 1). It must be admitted that "bound" water has never been demonstrated in the erythrocytes. Anelectrolytes even seem to be adsorbed to the haemoglobin, it remains to be tried, how the electrolytes behave.

Ad 2) and 3). It is difficult to see how the difference in pressure between the watery phases of serum and cells can exceed 1 atmosphere as in the experiments at an outer pressure 2.3 times osmotic pressure.

Thus 4) at present seems to be the most plausible explanation.

DAVSON, DUKE-ELDER and BENHAM (1936) investigate the Donnan ratios in aqueous humour and serum and find that there is no reason to suppose that the activity coefficients will be the same in these media.

### Summary.

It is demonstrated as by other authors that the volume of the erythrocytes does not follow Boyle-Mariotte's law. At hypotonic pressure the discrepancy is not considerable, but at hypertonic pressure it corresponds to 15—20 % of the cell water being "bound".

The diffusion through the cell membrane of potassium, sodium and phosphate during the experiment is insignificant. The haematocrit values obtained are very reproduceable.

The intercellular fluid of the centrifuged blood cell mass is 1.7—3.8 % in normal blood, in hypotonic solutions it decreases and in hypertonic solutions it increases.

It was formerly shown by the author that lead makes the erythrocytes permeable to potassium. It is now shown that sodium can permeate as well, and that the two substances permeate with the same velocity. The potassium loss of the erythrocytes make them shrink considerably, and the haemoglobin concentration increases.



The osmotic pressure of the serum water and the cell water in normal blood and blood that has been poisoned is determined by K, Na, Cl and  $\text{HCO}_3$  analysis. An excess of ions is found in serum and this is increased by lead poisoning, and more than the amount of cell water is decreased indicating that the higher haemoglobin concentration increases the "bound" water.

In order to explain the problem of "bound" water 4 hypotheses are advanced and discussed.

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## Some Relations in Gastric Stimulation by Intravenous Injection of Histamine.<sup>1</sup>

By

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POPIELSKI (1920), KEETON, KOCH and LUCKHARDT (1920) and ROTHLIN and GUNDLACH (1921) discovered that  $\beta$ -imidazolylethylamine (histamine) had a powerful stimulating effect on the gastric glands when given subcutaneously. They failed, however, in stimulating the glands by injecting the histamine intravenously all at once. IVY and JAVOIS (1924) and GUTOWSKI (1924) when giving histamine intravenously over a period of 30 or 50 minutes, succeeded in getting a copious flow of gastric juice. They realized that not only the amount of histamine given, but also the time was of importance.

TEORELL (1932 and 1933) was able to show mathematically that in POPIELSKI's experiments the histamine was probably destroyed in the blood before causing any stimulation of the gastric glands. He proved that a constant histamine concentration in the blood must be maintained if a stimulating effect is to be the result. Such a constant level is reached only by a slow *continuous, intravenous administration*. Experimental studies, which can be taken as support for this theory, are made by TEORELL (1937) and EMMELIN, KAHLSON and WICKSELL (1941).

In order to investigate the gastric secretion under the conditions given by a continuous, intravenous administration an apparatus has been built after the model given by LINDGREN (1943). By this apparatus a syringe is driven at a slow rate, which can be varied over a wide range. Histamine of different concentrations can be injected in that way.

<sup>1</sup> All figures in this paper are expressed in terms of histamine-dihydrochloride.

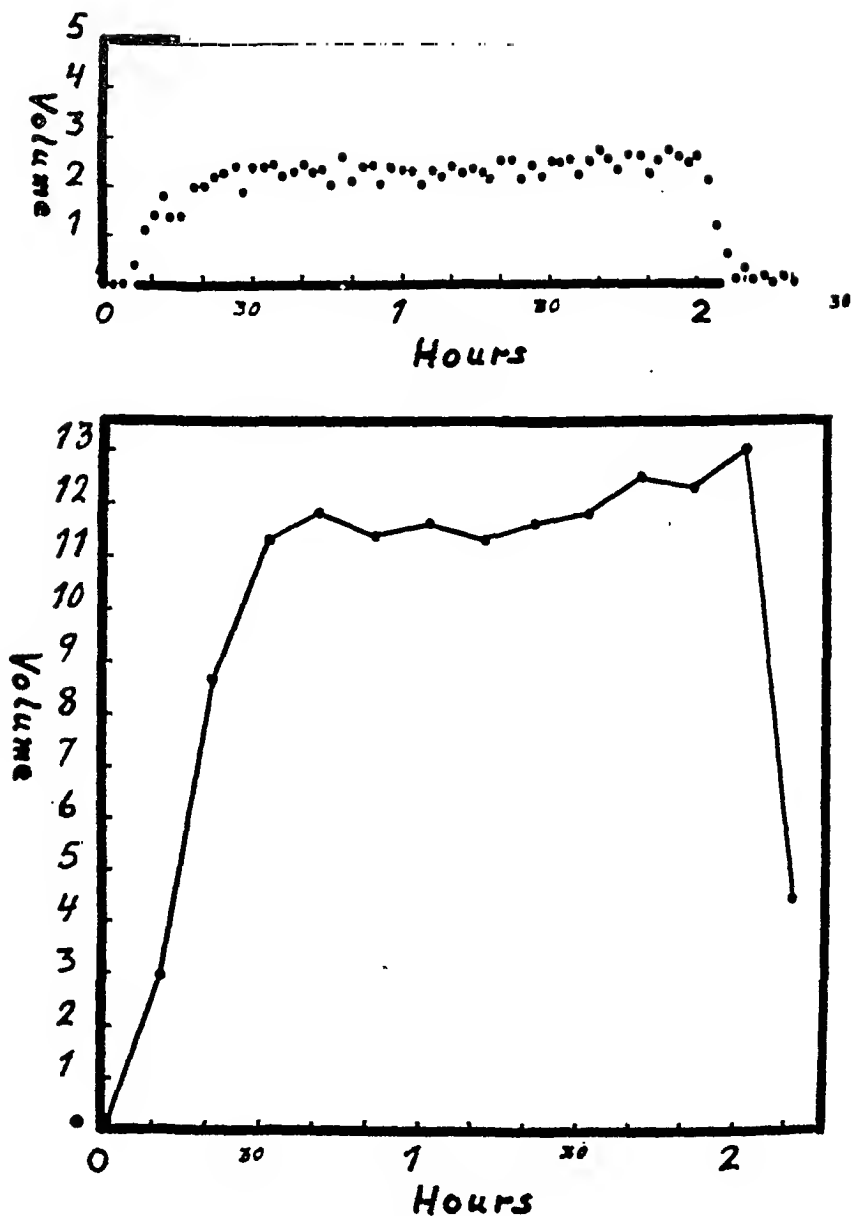


Fig. 1. Gastric secretion after continuous intravenous injection of histamine. The injection is started at the time zero and continued at a constant rate. At 2.00 hours the injection is interrupted. Volume in ml/2 minutes.

- a) The amount secreted in 2 minutes.  
 b) " " " " 10 "

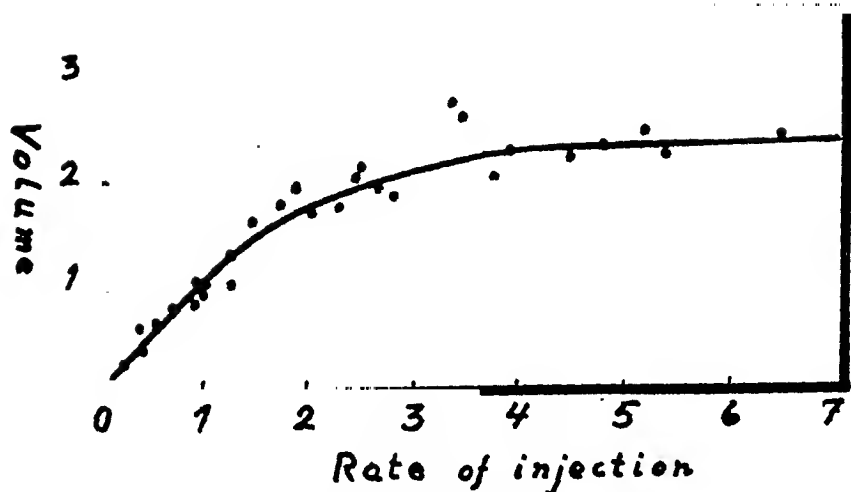


Fig. 2. The relation between the rate of injection (mg/hour) and the steady state volume secreted (ml/2 minutes).

The following experiments have been carried out on a dog (19.7 kg of weight) with a Heidenhain pouch. Before starting, the pouch has been washed out with warm saline. The injections have been made into a superficial vein of a leg.

The volumes secreted have been recorded *every 2 minutes*. After some four to six minutes the gastric juice appears but does not reach a steady state before twenty to thirty minutes. After that an almost constant level can be maintained for hours.

*Fig. 1* shows a typical case.

When the injection rate is changed a new constant level will be reached. *Table I* includes some different injection rates and the corresponding *steady state* volumes obtained in two minutes.

Putting these values into a coordinate system we get the *fig. 2*.

From this figure it is seen, that a maximal flow of gastric juice is easily obtained, a fact which ought to be kept in mind, when a secretagogue or an inhibitor substance is to be tested. *Differences in the stimulating or inhibiting effect can only be discovered, when the stimulation is submaximal.*

It is also seen, that there is no stimulation threshold for the histamine administered. The curve in *fig. 2* passes through the origo, i. e. even very small injection rates initiate secretion. (Here the histamine normally existing in the blood of a fasting animal is neglected.)

Further discussion and mathematical analyses of the results will appear later elsewhere.

Table 1.

*The relations between the rate of injection and the steady state secretion during 2 minutes.*

(The first column shows the concentration of histamine injected.)

Conc. of histamine pro mille	Rate of injection mg/hour	Amount per 2 minutes ml.
0.025 .....	0.10	< 0.1
0.025 .....	0.14	< 0.1
0.025 .....	0.18	0.2
0.10 .....	0.40	0.41
0.10 .....	0.40	0.60
0.20 .....	0.54	0.69
0.10 .....	0.68	0.82
0.25 .....	0.95	0.85
0.25 .....	0.95	1.08
0.30 .....	1.03	0.94
0.30 .....	1.03	1.04
0.33 .....	1.29	1.03
0.25 .....	1.30	1.34
0.20 .....	1.50	1.65
0.50 .....	1.78	1.82
0.10 .....	1.90	1.95
0.33 .....	2.06	1.75
0.30 .....	2.34	1.78
1.00 .....	2.50	2.07
0.33 .....	2.53	2.16
1.00 .....	2.70	1.96
0.75 .....	2.85	1.91
0.75 .....	2.85	2.11
0.50 .....	3.40	2.76
0.50 .....	3.50	2.67
1.00 .....	3.80	2.06
0.20 .....	3.92	2.34
1.00 .....	4.50	2.25
0.25 .....	4.80	2.36
1.00 .....	5.20	2.49
1.00 .....	5.40	2.30
1.00 .....	6.50	2.40

### Summary.

By a slow continuous, intravenous administration of histamine the gastric glands are stimulated, and the relations between the stimulation and secretion are discussed.

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## Growth Limiting Factors of Tissue Cells in vitro.

By

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It is a well known fact that the growth of tissue cells *in vitro* stops even under the most favourable conditions long before the surface of the culture medium is covered with cells. A size of 5 mm in diameter, often met with at the end of the growth in a D<sub>3</sub> Carrel flask (30 mm in diameter), makes only 1/36 of the surface. The disproportion between terminal size of the cultivated tissue and the amount of medium (1—200,000 by weight) seems to indicate that the tissue in question has at its disposal a rather limited "span of life" under these experimental conditions. This remarkable fact gave us an occasion to analyse the problem in which an important biological principle seems to be involved. This investigation might also disclose why a few scattered cells or a single one are unable to multiply and give rise to new cell colonies, as do single bacteria.

In this paper experiments are described which throw light upon the factors operating in the cessation of growth.

1. When the growth of an explanted fragment of tissue comes to a stand-still in a culture medium which is about 200,000 times the weight of the tissue, it seems improbable a priori, that the arrest of growth can be attributed to accumulated waste products of the tissue cells or lack of nutrient substances which the following experiments confirm. The cause is to be looked for within the tissue itself if anything rather than outside in the medium.

In flask cultures containing mesenchyme cells which had multiplied under optimal conditions until growth ceased, a new tissue fragment belonging to the same strain of cells was inserted in that part of the coagulated plasma which was free of cells. Area measurements showed that while the growth of the original tissue had ceased, the tissue later implanted continued to grow normally and reached a considerable size. Still more instructive was another experiment in which we were able to show that a coverslip culture which has ceased to grow resumes its activity when excised and reimplanted in another part of the same plasma drop.

This shows that the act of transplantation itself must be the immediate cause of the "rejuvenation" of the tissue.

An example from another set of experiments is this. Under identical conditions one tissue fragment was placed in one flask, while four other fragments belonging to the same strain were placed in an identical medium in another flask. At the end of the experiments, when the growth had ceased, the surface areas were measured. The final area of growth of the single fragment amounted to 1,793 units, while the sum of the total areas of the four fragments amounted to 5,460 units. Thus the total area of the four fragments exceeded by far the area which the single fragment attained under identical conditions. Finally it is also possible to obtain a growth which covers the entire surface of a flask by introducing numerous small bits of tissue in the medium. It therefore seems natural to assume that *the main cause responsible for the growth limitation is not to be found as a defect of the surrounding medium, but in an intrinsic factor in the tissue itself.*

Our attention should therefore be directed towards the so-called "residual growth energy" of the tissue; a concept which was introduced by CARREL and co-workers. According to these authors the "residual growth energy" of a tissue *in vitro* is measured by the rate of proliferation in a protective medium, a physiological salt solution or a plasma medium not containing embryo extract. Further, the residual growth energy is related to the inherent energy, so that changes in the latter may be derived from changes in the former.

EARLE and THOMPSON (1930) discovered long ago that the size of the terminal area of growth depends on the initial size of the explanted tissue fragment. Without knowledge of their paper EPHRUSSI and TEISSIER (1932), in measuring the residual growth



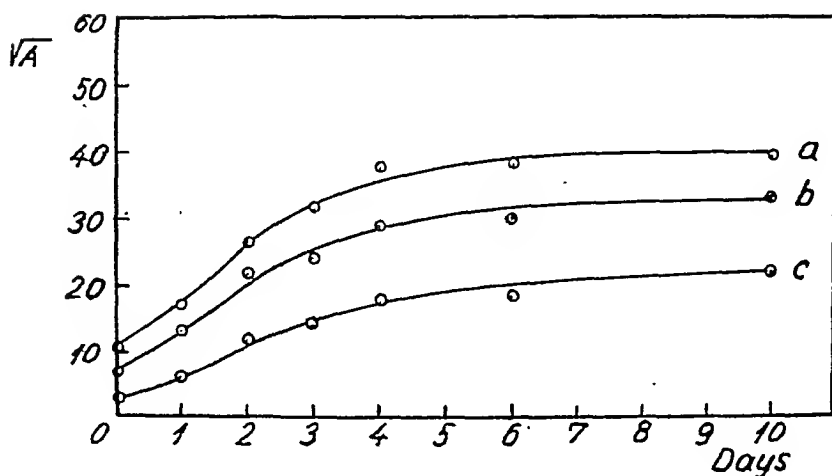


Fig. 1.

energy according to CARREL, also found that the residual growth depends not only on the amount of growth substances and nutrient material which the particular tissue contains at the beginning of the experiment, but also on the size of the tissue fragment. This we have been able to confirm and furthermore demonstrate, contrary to the interpretation of EPHRUSSI, that it also holds good when the medium contains ample amounts of growths promoting substances. Fig. 1 illustrates an experiment of this kind (Exp. 6,665). A culture was cut into fragments of different sizes and the fragments transferred to identical media on coverslips. The smallest fragment ceased to grow at an earlier time than the sister cultures with larger initial areas. This clearly demonstrates that the cessation of the growth is independent of the composition of the medium and is in no way due to an accumulation of waste products or to lack of nutrient material in the part of the medium not invaded by the cells. The explanation may be that the tissue itself contains important substances which are not present in the culture medium and which may be supposed to be consumed by the cells during a single growth period. How would it then be possible to explain the re-appearance of these substances, which are supposed to be completely consumed on transferring the tissue to a fresh medium? As we shall see later, this explanation is nevertheless the right one.

The theoretical considerations of BUCH ANDERSEN and FISCHER (1928) show that the growth (increase of radius with time), pro-

ceeds according to a typical S-curve, which gives evidence that an inhibitory factor  $a$  in the growth formula:

$$\frac{dr_t}{dt} = k - ar_t \quad (r = \text{radius, } t = \text{time})$$

is already active from the beginning of the growth.

The residual growth energy according to CARREL is an expression for the amount of a growth substance or of a simple reserve of nutrient material contained in the cells, which might partly explain why a larger fragment will continue to grow and cover a bigger area than a smaller one under the same conditions of experiment. But since this also holds true just the same under conditions for optimal growth, it indicates a mechanism which must be entirely characteristic for the growth of tissue cells *in vitro*, regardless of whether the medium contains abundance of adequate nutrient material or not.

The mathematical formulation of the growth indicates that all growth-curves are sigmoid curves and since the cessation of the growth can not be explained by an inhibitory effect due to accumulation of waste products or lack of nutrient material in the medium for which we have evidence, the S-shaped growth curve obtained even under optimal conditions must be the expression of a true residual growth. In other words this indicates that one or several important substances may be exhausted during the growth *in vitro*. This theory actually explains why a larger fragment of tissue will reach a bigger terminal area of growth than a smaller one.

On the basis of the theoretical consideration quoted which BUCH ANDERSEN and FISCHER advanced about 16 years ago, that *the inhibition of growth increases with the linear extension of the tissue* — it was suggested that the cause was to be found in diffusion processes. It now seems possible to confirm this idea by means of experiments.

2. Before we proceed with the main problem to be dealt with here, it is necessary to include a short description of the mechanism of tissue growth under the special conditions prevailing *in vitro*.

The first question is: What is the immediate cause for the radial migration of cells from the explanted tissue out into the surrounding medium and what are the conditions, which determine the radial-symmetrical growth-polarity of the culture?

When the tissue fragment is removed from the organism and placed in the culture medium, the conditions for the tissue are radically changed. The migration of the cells from the closely packed tissue fragment indicates this. The composition of the limited medium *in vitro* must vary incessantly, due to the waste products which diffuse out of the cells. With the tissue as centre a radial concentration gradient of metabolic products will arise which is responsible for the migration of the cells out into the medium.

The migration is the manifestation of a one-sided influence to which the cells are subject. The cells are not able to eliminate the waste products in any other way than "fleeing" from them. BURROWS (1913) who many years ago suggested this mechanism, says: "The cells radiate out on all sides like the light waves from a central illuminating body." When the cells are in a field of force, in this case a field of diffusion, they are affected one-sidedly by chemical substances, hence the cytoplasm will respond differently on the two sides, and each cell will adjust itself in such a way that symmetrical parts of its surface will be met by the diffusion line at the same angle. Migration of the cells makes the main contribution to the areal increase of the tissue and the increase in mass is provided for by cell multiplication which makes up only a small fraction of the "growth process". It is therefore probably in the nature of the migratory activity of the cells that we may primarily look for an explanation of the early cessation of growth in a large excess of culture medium.

Since the radial migration of the cells from a fragment of tissue irrespective of its shape, results in a circular cell-colony wherein the mitotic activity of the cells reaches a maximum within 24 hours, a gradual decrease of cell density will occur towards the periphery of the tissue on account of the divergent migration of the cells and the decreasing rate of cell division. The circular shape must therefore be the result of a state of equilibrium. There is much to indicate that the distribution of the cells in the colony or the "degree of disaggregation" is a decisive factor in determining the cessation of growth. This growth balance may roughly be expressed in the following scheme:

Cell association—cell migration—cell multiplication—cell dis-		
		aggregation
metabolic minimum)	(metabolic maximum)	(degeneration)

According to this, *the terminal size of a given culture will always depend upon the number and density of cells in the explanted tissue, regardless of the composition of the nutrient medium.* Only with an extreme excess of fresh embryo extract does it seem possible to break this rule. This will be mentioned later. From this we may conclude that, except with extremely large amounts of embryo extract, the size of the colony of cells under identical conditions is almost proportional to the size of the explanted tissue fragment.

3. On the basis of the available facts, much speaks in favour of the idea that the terminal dissociation of the cells in the marginal zone of growth is in some way or other the immediate cause of the cessation of growth. At any rate changes in the great bulk of the surrounding medium, such as an exhaustion of nutrient substances or a saturation by metabolic waste products can not be responsible.

At the time when the marginal cells of the tissue evidently become scattered as the result of the radial spreading, they undergo visible changes which indicate that they are suffering from an inadequate milieu and in that respect they contrast with the cells in the intermediary and the central zones of growth, which have a normal appearance. This phenomenon is familiar to those working with tissue cultures. One could not have expected that in particular the dissociated cells in the most peripheral part of the tissue would be the first to suffer a vacuolar and fatty degeneration. It has been difficult to understand just why the most peripheral cells in the culture should die first, since they are immediately exposed to the fresh part of the medium. SZANTROCH (1932) was the first to call attention to this puzzling fact, which now seems to be well explained. Very often it can be observed that only one or two cells, at the very end of a long, thin chain of cells protruding out into the medium from the main colony, are very much vacuolised. The most peripherally located cells in the culture are really the oldest — but this does not explain why just these cells should suffer most from a deficiency, when they are surrounded practically on all sides by a perfectly fresh culture medium.

That it is in the marginal zone of growth that the cells are scattered and degenerating shows that the cells, on the average, are not able to migrate further out without being irreversibly damaged under the experimental conditions.

Doubtless it is in the conditions among the "dissociated" cells in the periphery that we must look for the actual "barrier" to

additional cell displacements in outward direction. When the cells reach this stage of aggregation they become damaged, undergo the above named degeneration and die. PARKER (1936) reported interesting experiments in which he was able to cultivate a single colony of tissue cells in the same CARREL-flasks for more than one year. He described how in the peripheral zone of the tissue, degeneration and death of the cells resulted in a compensatory migration from the interior of the solid, central part of the tissue. This phenomenon of marching on of the cells within the same spot gives a good illustration of what really happens in the normal culture, when the areal spreading is nearly at an end.

All the observations made (for more details see FISCHER (1946)) indicate that the conditions for the maintenance of cells in the central part, representing the great mass of packed cells, are very different from those in the peripheral zone of more or less scattered cells. In other words, the cells in the compact mass of the tissue in the central part and in the intermediary zone are separated from each other by only very narrow intercellular spaces in contrast to the very large spaces between the cells in the marginal zone of growth.

The question is: How is it possible for scattered cells to act as an obstacle to the expansive growth of the cells out into the free culture medium? We have shown, by introducing numerous small fragments of tissues into a CARREL-flask, that it is possible to have the entire surface of the medium covered with cells. It is also possible to obtain, from a single fragment of tissue, a growth covering the entire surface of a CARREL-flask (BAKER 1939). This author does not say how often this experiment can be reproduced. A big growth like this could only be obtained by using a very large quantity of embryo extract (66 p. ct. in the medium) which perhaps indicates that the extract may contain an important factor present only in a very low concentration.

4. In an earlier publication (FISCHER and co-workers 1945) experiments were described with the purpose of testing whether the difference in behaviour of the cells in the marginal zone and in the central part of the culture could be explained by differences in oxygen tension. The experiments showed, however, that the influence of the oxygen tension was not responsible.

Investigations on the significance of amino acids, and other low molecular substances (FISCHER 1941, 1942) contained in the blood plasma, for the maintenance and growth of tissue cells,

showed clearly that the lag is a most critical period to be overcome when the medium does not contain enough of the right intermediary products. All the cells in the culture are filled with big vacuoles when the medium is insufficient. If the cells do not get a good start they will slowly disintegrate. It is well known from the study of bacterial growth, that the lag is a period during which essential intermediates are built up (HINSHELWOOD 1944), and that it may be shortened by the addition of a filtrate from the appropriate growing culture.

Since the migrating tissue cells in the culture do not distribute themselves on the entire surface of the medium, but stick together forming a closed community in which the cells are more or less connected to each other by protoplasmic bridges, an extensive growth covering the entire surface of the medium can probably only be obtained when a very large amount of the important intermediates can be supplied. So far this has only been accomplished by BAKER (1939), who was unable to give any real explanation. The embryo juice naturally must contain great amounts of intermediates, which may be responsible for the large growth obtained by this author. It is no doubt of importance to use the embryo-extract immediately after it has been prepared, otherwise the intermediates may mutually react or be adsorbed on to the easily denaturing proteins, with marked reduction in their effect on the cells. It is possible to obtain a very large growth from a single fragment of tissue by using huge amounts of embryo juice and since the growth otherwise is limited, the final size being proportional to the size of the explanted tissue, it seems as if the latter phenomenon may be of an intrinsic nature.

The question is then: Is there any connection between these two phenomena, the unlimited growth obtained by BAKER and the limited growth under the common conditions, that is, are the substances present in the embryo extract identical with those contained in the tissue fragment itself which seem to disappear during a single growth period of, let us say, 3—4 days? If they are identical, how is it then possible for "the exhausted tissue fragment" to recover and grow again, after a transfer to a new medium?

According to the nature of the growth of the cells *in vitro*, the radial spreading from the tissue fragment reaches a maximum at the time when the growth is about to stop. At this time the cells undergo a vacuolar and fatty degeneration and disintegration fol-

lows. Between the spreading of the cells and their degeneration there must be a causal connection. Evidence for this is established by the fact that the cells migrating from very small fragments of tissue suffer an early degeneration and disintegrate rapidly.

It is possible to prevent the rapid degeneration of the cells and thereby demonstrate that its immediate cause actually is the spreading of the cells. Two fragments of tissue were placed in the same medium, with a distance between them such that their zones of growth would meet about the time the peripheral cells would undergo vacuolar degeneration. The peripheral cells of the two colonies showed the usual signs of degeneration, with the exception of those in the space between the two colonies where the cells were about to meet.

These facts suggest that strong social forces bind together the tissue cells which are unable to survive unless they are allowed to communicate almost directly with other cells. Thus it becomes evident that *the scattering of the cells in the marginal zone of growth must be the primary cause of their degeneration and disintegration*. It is a fact that the cells in the central part of the tissue, where they are closely packed together in several layers, live much longer than those in the periphery. The only explanation for this paradoxical behaviour is that the cells in the marginal zone are compelled to take up an unfavourable situation, characterized by an interruption of the social connections between the cells. When the population of cells decreases in the marginal zone of growth, they will be separated by larger open spaces which will lead to an escape by diffusion of important intermediates from the tissue. From the narrow interstitia in the closely packed central part of the tissue escape by diffusion is much retarded.

This explanation of the behaviour of the cultivated tissue cells *in vitro* may lead to an extension of our knowledge of some of the most characteristic features responsible for the perfect social properties displayed by the cells in higher organisms. It will then probably be possible to enter an important new field of investigations which hitherto has been inapproachable and rather obscure, namely the regions beyond the smallest units of the blood vessels, the intercellular spaces. The method of tissue cultivation may here have an opportunity to give new information. The intercellular space in general is probably the theatre where most important interactions of the cells take place. The flow of

the intercellular fluid must be relatively slow and the concentration of intermediates rather high.

Many years ago one of us (FISCHER 1923, 1927) called attention to the phenomenon that single isolated cells scattered in a culture medium with ample amounts of embryo extract are unable to multiply and give rise to new colonies of cells as do the bacterial organisms. It is therefore by no means impossible that the explanation for this is in principle the same as is responsible for the disintegration of the peripheral cells in a culture when growth has ceased. It was suggested at that time that the cytoplasmic bridges between the individual cells in the organisms, as well as in the culture, represent an important organ by which substances (desmones) or stimuli of various kinds might be exchanged. It would be worth while to take up again this question for further consideration. On the other hand cytoplasmic bridges from cell to cell may be a physiological system of another type than that of the interspace of the cells, both of which provide for the maintenance of a strong social system among the cell elements.

From experiments on cultivation of tissues *in vitro* we now know with certainty that the composition of the intercellular fluid is different from that of the plasma medium used for the cultivation of the cells. When to this medium a large excess of embryo juice is added, as shown by BAKER, the cells will be able to retain their juvenescent stage and able to cover the entire plasma surface with young cells.

### Summary.

1. The mechanism of tissue growth *in vitro* is summarized taking in special consideration the factors which bring about the discontinuation of the growth. A special attention is called to the remarkable fact that under the ordinary conditions of experiment the terminal area of growth is almost proportional to the size of the explanted tissue.

2. Neither the waste products produced by the cells nor lack of food can be made responsible for the arrest of the growth during a single cultivation period.

3. The radial-symmetrical growth of the tissue is the result of the migration of cells in part those already present in the tissue as well as those formed by multiplication.



4. At the end of the growth period only the cells in the marginal zone undergo a vacuolar and fatty degeneration which leads to their disintegration. This indicates that the cells suffer from a deficiency due to the increasing distance between the cells, a situation which brings about that intermediary metabolic substances escape from the cells by diffusion out into the medium.

5. The main cause for the limitation of the growth is not to be found in the formation of an inhibitory factor but in an escape from the dilated interspace of important intermediates, an explanation which was foreseen many years ago by BUCH ANDERSEN and FISCHER in their formulation of the growth curve, a theory for which evidence now is available.

6. The results are discussed as to the significance of the interspace and the cytoplasmic bridges between the tissue cells for the maintenance of the strong social relations of the cells.

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## Determination of Potassium in Urine by Means of Periodic Acid.

By

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The majority of the methods of quantitative determination of potassium in animal fluids, recommended in the literature, are based on the methods known from inorganic analysis of precipitation by means of chloroplatinic acid or sodium cobaltinitrite. Lately, the use of organic precipitation substances and of spectrographic analyses has been proposed in this connection (FREDHOLM 1936, THOMSEN and LEE 1937). However, these methods do not seem to have been widely adopted. The cobaltinitrite method has generally been preferred in routine physiological and biochemical practice, in some cases supplemented by silver salt. In the urological technique, it can, in spite of its defects, nowadays probably be regarded as predominant. Its greatest drawback lies in the fact that the composition of the precipitate varies according to external circumstances, such as the temperature, or according to the concentrations of the reactive substances. Most authors, therefore, advocate this method for clinical practice in the form of a comparison, generally by colorimetric means, between the precipitate from the test and from a standard solution of known potassium content (BREH and GAEBLER 1930, DUKES 1939). However, this procedure may be regarded as highly empirical and the sources of error inherent in it are numerous.

Considering the significance of a quantitative determination of potassium in the urine, in general physiology as well as at metabolic investigations and urine analyses included in the clin-

ical routine, a more appropriate and handy method has seemed desirable. Such a method is, in fact, at our disposal thanks to the periodic acid now in the market.

The precipitation of potassium as a periodate has been suggested by GREATHOUSE (1917) as an analytical method. This process was later studied by HILL (1928) and further developed by WILLARD and BOYLE (1941) into a determination technique on inorganic material. On the other hand, it has apparently not been employed in biochemistry. Still, by observing certain precautions, this method has been found suitable both as regards animal tissues, fluids and minor organisms (*e. g.* fish spawn and planeton). As a rule, certain preparative treatments of the original material are called for. However, at an estimation of potassium in the urine, the preparative treatment may be simplified to a great extent under certain circumstances, without a deterioration in the accuracy of the determination.

The method thus obtained is quick and simple, not requiring any outfits other than those of ordinary laboratory equipment. However, certain precautions should be observed, above all, with regard to the tendency of the periodate to become reduced by different substances. The isolation process cannot be completed in an aqueous medium owing to the solubility of the potassium periodate (approx. 0.5 % at room temperature). Furthermore, the precipitation should be performed in such a way as to prevent the inclusion of by-products in a form that may interfere with the result of the determination.

Under normal conditions, the following procedure has been found advisable: 2 ml of the filtered or centrifuged urine are transferred by means of a pipette to a sintered glass filter crucible (porosity No. 3 or 4) of an approximate capacity of 30 ml, tightly stoppered from below by means of a carefully adapted rubber plug and supplied with 1 or 2 ml of a concentrated aqueous solution of periodic acid. Then 20 ml of a compound of equal parts of 96 % ethyl alcohol and ethyl acetate are added and the ingredients are thoroughly mixed. Should the mixture become milky or opalescent, more of the periodic acid solution is required. The filter crucible, the contents of which must be stirred repeatedly with a platinum thread or a narrow glass rod, is left in a refrigerator for 30 minutes at a temperature of 0°. After this time, the rubber plug is extracted and the crucible is applied to a suction aggregate. When the liquid has been removed, the

precipitate is washed 4—5 times with a few ml of ethyl acetate, which has been cooled to 0—4°. Care should be taken that no precipitation substance remains at the upper part of the walls of the filter crucible.

The obtained precipitate does not generally consist only of potassium periodate ( $\text{KJO}_4$ ) but also contains a varying quantity of other components, according to the nature of the urine.

Thus, the weight of the precipitate as a rule becomes greater than corresponds to the potassium content of the test. Gravimetric determination would therefore be misleading. The precipitated by-products, on the other hand, do not affect the volumetric determination by titration with arsenite, which may be carried out as follows:

The filter crucible, which has been well cleaned on the outside and along the edges, is placed together with its contents in a capacious glass vessel (volume approx. 350 ml). About 100 ml of a buffer solution is added, prepared from equal weights of boric acid and borax in 30 parts of distilled water.<sup>1</sup> The precipitate will in this way easily be dissolved. A few grains of potassium iodide are then included. The periodate is reduced to iodate and the liquid is stained by liberated iodine. Its quantity is determined by titration with a 0.1-normal arsenite solution.

This is prepared by dissolving 4.945 g of pure arsenious oxide ( $\text{As}_2\text{O}_3$ ) at 80° in approximately 400 ml of water to which 10 g of water-free soda has been added. Carbon dioxide is then introduced to a neutral or weak acid reaction and the solution is diluted to a volume of 1,000 ml.

The quantity of potassium in mg is obtained by the multiplication of the required arsenite solution in ml by the factor 1.955.

The accuracy of this procedure was checked with regard to normal urine of man and certain mammals (*Bos*, *Cavia*). The control tests were carried out on the basis of two different criteria. Firstly, the results were compared with determination of the potassium content of the ash from one and the same urine sample. Secondly, the potassium content of the urine was varied by the addition of established quantities of potassium salts, and the suitability of the method of accounting for these variations was examined.

A few examples are given here in illustration of this. As regards the ash analyses, recorded in Table 1, the relation between the

<sup>1</sup>  $P_H$  = approx. 7.5.

Table 1.  
Determination of potassium in urine by means of periodic acid.

Date	Analysis of the ash of 2 ml urine						Direct analysis of 2 ml urine				
	Weight of precipitate	Required amount of 0.1 N arsenite	Calcul. equivalent Wt of precipitate	Potassium found		Weight of precipitate	Required amount of 0.1 N arsenite	Potassium found titrimetrically	Calcul. weight as periodate	Surplus weight of precipitate	
				Gravimetrically	titrimetrically						
1	2	3	4	5	6	7	8	9	10	11	
7/6	39.6 mg	3.5 ml	113.1	6.7 mg	6.8 mg	49.0 mg	3.4 ml	6.7 mg	39.1 mg	9.9 mg	
13/5	38.5	3.3	116.7	6.5	6.5	47.4	3.3	6.5	38.0	9.4	
17/6	40.0	3.4	117.6	6.8	6.6	40.8	3.4	6.6	39.1	1.7	
20/5	28.6	1.8	158.9	4.5	3.5	32.2	1.6	3.1	18.4	13.8	
20/5	22.0	1.7	122.2	3.7	3.3	33.0	1.7	3.3	19.6	13.4	
13/6	57.0	5.0	114.0	9.7	9.8	67.0	5.1	10.0	58.7	8.3	
11/6	—	2.5	—	—	4.9	42.6	2.5	4.9	28.8	13.8	
3/5	—	1.0	—	—	2.0	19.2	1.0	2.0	11.5	7.7	
Urine of Cavin	42.0	3.6	116.7	7.1	7.0	42.0	3.6	7.0	41.4	0.6	
Urine of Bos	123.6	10.7	115.6	21.0	20.9	133.4	11.3	22.1	130.0	3.4	

<sup>1</sup> Incineration incomplete.

absolute weight and the oxidizing capacity of the precipitate is fairly constant and consistent with the presupposition that the equivalent weight of the precipitated substance is identical with that of potassium periodate  $\left(\frac{230}{2}\right)$ . Thus, the gravimetric estimations of the potassium content harmonize with the results obtained by titration (cp. columns 5 and 6). The complete incineration of the urine is a prerequisite in this connection. When the incineration is incomplete the gravimetric method usually gives too high values (cp. the first operation of the 30/5).

In the directly performed analyses (*i. e.* without incineration) of the same urine samples, no constant relation is found between the gravity and the oxydizing capacity of the precipitate. In all these experiments, the precipitates exhibit an excess weight which has indeed quite a variable magnitude (cp. column 11). The potassium content of the urine cannot therefore be estimated gravimetrically. On the other hand, the values obtained in the iodometrical titration harmonize well with the results of the ash analyses (cp. columns 8 and 9). Evidently the surplus gravity is caused by substances not interfering with the titrimetrical process. Thus, the titrimetrical determination of potassium in this connection is possible without the complications generally involved in the incineration process.

In the experiments, recorded in Tables 2 and 3, at first a titrimetrical determination of the proper potassium content of the urine sample was performed. The results were checked by means of determinations of the potassium content of the ash. Then, the composition of the urine was modified by the addition of solid potassium sulfate or 0.2 N aqueous solution of  $K_2SO_4$ .

Table 2.

*Titrimetrical determination of potassium separated as periodate from human urine with and without addition of various quantities of potassium sulfate.*

	Natural urine	Ash of urine	Urine + $K_2SO_4$	
Quantity of sample .	2.0 ml	2.0 ml	2.0 ml	2.0 ml
Added potassium . .	—	—	15.6 mg	7.8 mg
Found , . .	4.9 mg	4.9 mg	20.7 mg	12.9 mg
Calculated , . .	—	—	20.5 mg	12.7 mg

In the first case (Table 2) 0.01 and 0.005 mols of the salt were dissolved in 100 ml of urine. 2 ml of each solution were examined. The values obtained were but slightly higher than the theoretical values.

Table 3.

*Titrimetrical determination of potassium separated as periodate from human urine with and without addition of various quantities of 0.2 N solution of potassium sulfate.*

	Natural urine	Ash of urine	Urine + 0.2 N solution of $K_2SO_4$	
Quantity of urine . .	2.0 ml	2.0 ml	1.0 ml	1.5 ml
Added potassium . .	—	—	7.8 mg	3.9 mg
Found , . .	2.0 mg	2.0 mg	8.6 mg	5.3 mg
Calculated , . .	—	—	8.8 mg	5.4 mg

In the second case (Table 3) the urine was modified not only with regard to its content of  $K_2SO_4$  but also concerning its degree of dilution. The results of the analyses were a trifle lower than the theoretical values.

### Summary.

A procedure for the quantitative determination of potassium based upon the separation of this metal as a periodate was applied to biological materials. It was found to be of particular value in urological practice, since no preparative treatment of the urine was required. The precipitate holds certain by-products which nevertheless do not interfere provided the determination is accomplished by means of iodometrical titration.

A procedure fitted for determination of the potassium content in urine samples of 2 ml was reported. The accuracy of this procedure was tested on the normal urine of man and certain mammals. Some control tests were published in Tables 1—3.

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## Application of "Square Wave Analysis" to Bioelectric Studies.<sup>1</sup>

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During the past three decades alternating current conductivity measurements have been employed frequently as a mean of characterizing living cell and tissue "permeability". In general one has measured the same object in rapid succession with A.C. of several, different frequencies and the results have been expressed in terms of parameters of a so called "impedance locus diagram" according to GILDEMEISTER-LULLIES-COLE. (For a fuller treatment of the theory of alternating current measurements on biological objects refer to the reviews by LULLIES (1930), COLE (1933, 1936, 1940), VON MURALT (1935) and DUYFF (1942). Cf. also TEORELL & WERSÄLL (1945).) A typical locus diagram is Fig. 1.

A serious draw-back of the A.C. impedance method of this type is that a complete set of measurements takes a considerable time, several minutes or more depending on the number of frequencies required to yield a good locus diagram. During this time it may happen that the object changes its impedance properties more or less rapidly, hence the resulting diagram may be greatly in error. Very rapid impedance changes were observed for instance by COLE & CURTIS (1938, 1940) on electrical stimulation of the *Nitella* algæ or the *Loligo* nerve. These authors

<sup>1</sup> This work has been aided by grants from the Rockefeller Foundation and from the "Stiftelsen Therese och Johan Anderssons Minne Fond". I am also under great obligation to Mrs GUDRUN PERSSON-JEMT, M. K., for valuable assistance given in the course of this work.



had devised a special bridge method which enabled them to analyze the quick impedance alterations. Recently in our own attempts to record the transient effects of direct current flow on the isolated, surviving frog skin it was found necessary to abandon the usual

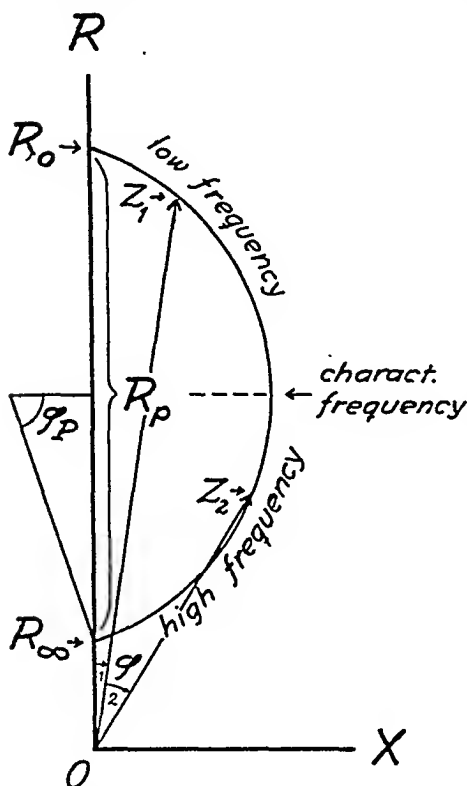


Fig. 1. Impedance locus diagram of a circuit of the type *a-b* of Fig. 4. —  $R_0$  = the steady state D.C. resistance (frequency = 0),  $R_\infty$  = the extreme high frequency resistance,  $R_p$  = parallel resistance,  $Z_1$ ,  $Z_2$  impedances at two intermediate frequencies with phase angles  $\varphi_1$ ,  $\varphi_2$ .  $\varphi_p$  is  $90^\circ$  only when the "polarization element" ( $C_p$ ) is a pure capacitance.

slow "point to point" A.C. method and instead search for a simple procedure capable of recording "instantaneous" impedance (or rather polarization) changes. Below we will describe "direct current transient analysis" or "square wave analysis" as a method fulfilling our requirements.

Among biologists already LULLIES (l. c. p. 1257) pointed out the theoretical connection between the investigations employing sinusoidal currents and those using "rechteckige Stromstöße", although he never seems to have attempted any detailed analysis in this respect. The same is the case also with COLE (1936, p. 75), who in a general way stresses the possibility of employing "transient analysis" for impedance measurements by aid of Fourier harmonic mathematics. MURDOCH & ZIMMERMAN discussed somewhat more in detail these questions in connection with electrode polarization studies. The actual impetus, however, to a practical use of "transient analysis" has recently come from the television radiotechnique, where the performance of video

amplifiers has been characterized by "square wave testing" (SWIFT; WALKER; HOADLEY & LYNCH; EMERY and others. For the present paper the practical schemes of BEDFORD & FREDENDALL have been of great importance). The aims of the television engineers and the biologists are, however, rather different and therefore this paper attempts to apply and modify the engineering technique to the requirements of the biologists.

## The Theoretical Relations between the Responses to Sine Waves, Square Waves and Direct Current Transients.

1) *Sine wave response:* — If a pure sine wave voltage is impressed on a complex impedance, as for instance a biological object with the equivalent circuit of Fig. 4, the steady state output voltage<sup>1</sup> will still remain sinusoidal but changed in amplitude and phase (cf. Fig. 2) to values varying with the fre-

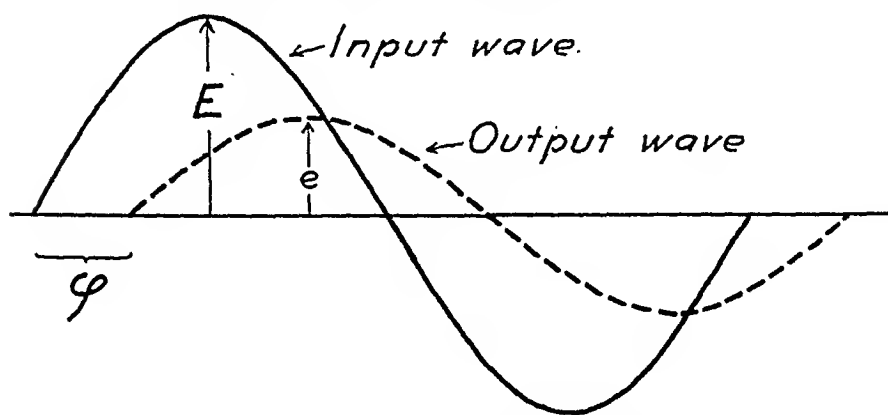


Fig. 2. Relations between a sinusoidal input voltage ( $E$ ) and the output voltage ( $e$ ) in a complex circuit.

The output wave is still sinusoidal but the phase is shifted in  $\phi$  degrees (negative sign in a circuit of the biological type, Fig. 4).

quency employed. This is best illustrated by vector representation<sup>2</sup> as in Fig. 9. From the *voltage vectors* it is in general possible to calculate the corresponding *impedance vectors* and thus the commonly used "impedance locus diagram" of Fig. 1 is obtained (cf. p. 236). Apparently each frequency point on such a diagram represents a separate determination.

2) *Square wave response:* — If a periodical square wave is impressed on the same circuit the output voltage obtains a different wave form and amplitude, the squares will be "distorted" (Fig. 3). The phase relation between the input wave and output wave will, in the type of circuit considered, remain as marked in

<sup>1</sup> The term "output voltage" is used here in conformity with the practice in the radio-technique when dealing with four pole circuits. In this case "current" would be the more adequate term.

<sup>2</sup> For the use of vector algebra and the "symbolical method" of calculations of A.C. circuits reference may be given to, for instance, SMITH, FRAENCKEL, HAUFFE or SCHÖNHOLZER.

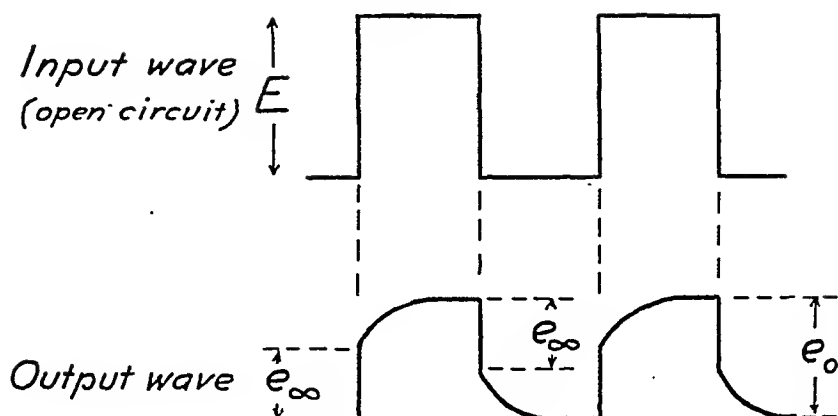


Fig. 3. Relations between a square wave input voltage ( $E$ ) and the output voltage ( $e$ ) in a biological circuit.

The output wave is "distorted", the phase relations between the steep parts remain, however, unaltered.  $e_0$  and  $e_\infty$  refer to steady state voltages of zero (= D.C.), and infinite ( $\infty$ ) frequency respectively.

Fig. 3. However, by a closer analysis of the input and output waves one can deduct the responses also of sine wave voltages and eventually a complete impedance locus diagram can be constructed just as with the varying frequency A.C. method, but with the important difference that only a half wave of the output voltage, generally of a very short duration, has to be recorded, i. e. an "instantaneous" determination is thus possible.

This "square wave analysis" is based on the fact that the square wave consists of a multitude of sine waves of different frequencies, which are related to one another in a specific manner describable by a Fourier series. The "distorted" output wave, as observed on the cathode-ray oscillograph, is then a collective observation on the individual effects of all sine wave components making up the square wave. By aid of Fourier analysis, however, the output wave can be broken down into these separate components.

It is well-known from Fourier wave analysis<sup>1</sup> that a periodical, symmetrical square wave (peak to peak amplitude  $E = 2 E'$ ) can be resolved into a series of sinusoidal waves composed of a fundamental frequency component together with all its odd numbered harmonics, the amplitude of each harmonic component being inversely proportional to its harmonic number ( $n$ ). This Fourier series can be written as follows (here  $\omega = 2\pi \cdot \text{frequency}$ ):

<sup>1</sup> Cf. for instance SMITH l. c. p. 221, SWIFT l. c. p. 24.

$$f(t)_{in} = \frac{4E'}{\pi} \left[ \underset{\text{fundamental}}{\sin \omega t} + \frac{1}{3} \underset{\text{3rd}}{\sin 3\omega t} + \frac{1}{5} \underset{\text{5th}}{\sin 5\omega t} + \dots + \frac{1}{n} \underset{\text{nth harmonic (Eq. 1)}}{\sin n\omega t} \right]$$

The amplitude of any sine wave component of this input wave is  $e_n$  (input) =  $4E'/n\pi$  (Eq. 2) and because cosine terms are lacking, the phase angle is  $\theta_n$  (input) = 0.

The distorted output wave owes its peculiar shape to the change of both amplitude and phase relations which is exerted on the input components by the test object. The Fourier equation of the resulting symmetrical wave is

$$f(t)_{out} = a_1 \sin \omega t + a_3 \sin 3\omega t + a_5 \sin 5\omega t + \dots \\ + b_1 \cos \omega t + b_3 \cos 3\omega t + b_5 \cos 5\omega t + \dots \quad \dots \quad \text{(Eq. 3)}$$

Here the amplitude of the  $n$ :th (odd!) sine component is  $e_n$  (output) =  $\sqrt{a_n^2 + b_n^2}$  (Eq. 4) and the corresponding phase angle expression is  $\tan \theta_n$  (output) =  $b_n/a_n$  (Eq. 5).

From Eqs. 4 and 5 it becomes evident that the desired sine wave values, *i. e.* peak amplitude and phase angle, can be easily obtained when the "cosine" and "sine coefficients",  $b_n$  and  $a_n$ , of the Fourier series of Eq. 3 are known. The determination of these coefficients is the aim of numerous methods described as "Fourier analysis", "harmonic analysis", "wave form analysis" etc. and we therefore refer to the special books where these procedures are described (for instance MANLEY, see also ROTHE, who employed a graphical method closely related to the one to be described later in this paper).<sup>1</sup> The principle of the transformation of the Fourier coefficients into corresponding coordinates of an impedance locus diagram will be described on p. 246.

Although the "regular" Fourier analysis method is of a general applicability, it suffers from some serious disadvantages, namely that circuit constants can not be calculated at frequencies lower than that of the impressed input square wave and at higher frequencies can only those be evaluated which belong to 3, 5, 7, . . . times the fundamental frequency. As the results at the higher harmonics are less accurate, the number of useful points on the final impedance locus will be reduced to perhaps only three or four. In the following section the principles of another method will be outlined which overcomes these limitations.

3) *Transient response*: — The response, for instance of a tissue, to a sudden make or break of a constant direct current is closely related to that of an alternating square wave as described in the

<sup>1</sup> Recently Dr A. BROMAN has developed a graphical-numerical method specially adapted for our purpose which employs only six y-coordinates pro half period and corresponding derivatives of the output wave. This method will be published elsewhere.

previous section, in fact one can regard such a single abrupt "shock" as a part of a square wave of an infinitely long period (or infinitely low frequency). The Fourier wave theories can therefore be extended to be valid also for electrical responses to such stepwise, abrupt voltage changes (such responses are defined as "transients", the unit of the abrupt step voltage applied is usually called the "Heaviside unit function"). Roughly the steep front of the step can be regarded as the response corresponding to an A.C. sinusoidal wave of infinitely high frequency and the following constant, flat part as corresponding to zero frequency (*i. e.* direct current!).

The exact theories show, however, that a step or unit function can be resolved into an *infinite* number of sinusoidal wave components of *all* frequencies, the amplitudes varying inversely with the frequencies (the common Fourier series is then transformed into a "Fourier integral", the relevant mathematical treatment may be found for instance in BUSH; BRAINERD etc.; BEDFORD & FREDENDALL or EMERY). Besides by this multiple sine wave concept the transient responses can be mathematically treated in other ways, well-known is the Heaviside operational method (cf. BUSH etc.).

From a practical point of view it is important that the response to a single, abrupt rise in a *repeating* square wave is essentially the same as the response to the Heaviside step voltage, *provided* the period of the repeating half cycles is taken *long enough* to insure that the output wave approaches a substantially constant level during the latter part of each half cycle.

Thus it is evident that a *step input voltage, or a long period square wave, impressed on a circuit corresponds theoretically to the simultaneous application of sinusoidal waves of the whole frequency spectrum*. In other words, reversing the process and *breaking down the distorted output voltage curve into its sine wave components ought to yield exactly the same information concerning the circuit tested as the common "point by point" single frequency A.C. method*.

Repeating square waves are nowadays easy to produce by means of special generators and it is also convenient to visualize them on a cathode-ray oscillograph screen where photographic recording is possible. A practical procedure for the breaking up of the output waves into sine wave components will be described under heading 2 on p. 243.

# Practical Procedures of Square Wave Analysis.

1. *Outline of the experimental conditions:* — In order to make the description of the square wave analysis more concrete we may assume that our analysis concerns the circuit  $a-b$  of Fig. 4 (which

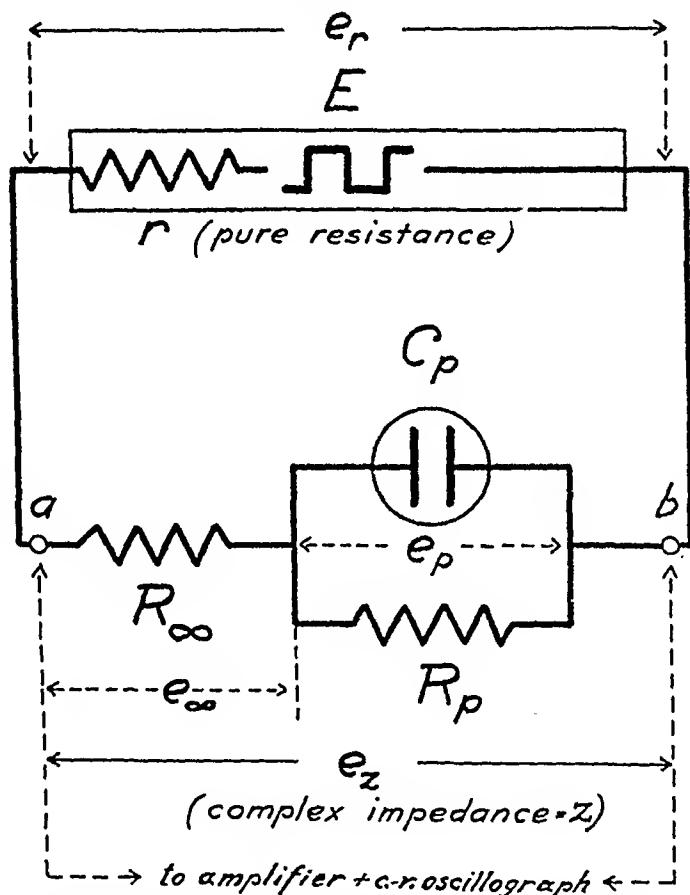


Fig. 4. Scheme of circuit conditions in "square wave analysis".

The test object extends between  $a-b$ . A square wave generator and an amplifier + cathode-ray oscillograph set up are connected in parallel over  $a-b$ . —  $R_\infty$ ,  $R_p$ ,  $r$  = resistances;  $C_p$  is a "polarization element" or capacitance (cf. Fig. 1). —  $E$ ,  $e_r$ ,  $e_\infty$ ,  $e_p$  and  $e_z$  refer to the voltage vectors of Fig. 9.

incidentally is an approximate equivalent scheme of many biological systems). A small step voltage (10—100 mV), or rather a long period square wave voltage from a pulse generator (total output resistance  $r$  ohms) is impressed on the leads  $a$  and  $b$ .

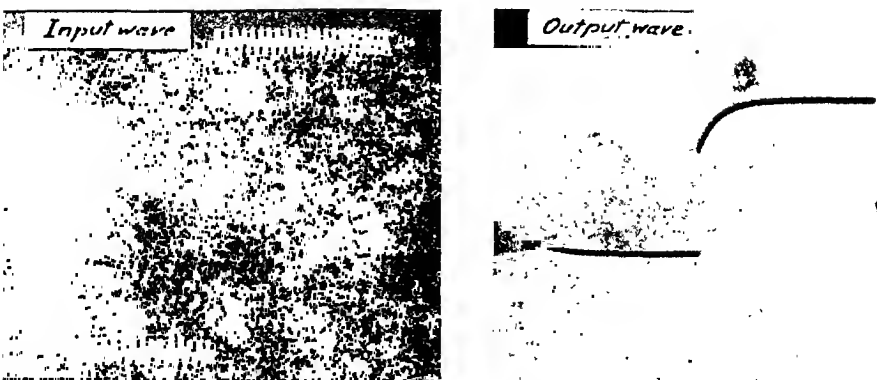


Fig. 5. Photographical records of an input square wave (left) and the output wave (right).

These records belong to the "model" circuit described on p. 249. Note that it is sufficient to record only a part of a wave. The superimposed timing frequency (3600 c.p.s.) is visible on the input wave.

In parallel, the same leads are connected over a suitable amplifier with a high input resistance (D.C. amplifier or an A.C. one with a good low frequency response) to a cathode-ray oscillograph preferably with a linear time sweep circuit. A distorted "output" wave as shown in Figure 3 or 5 can be recorded on the screen.

This wave consists of a symmetrical combination of two identical perpendicular parts of the relative length of  $e_o$  and two curved parts flattening out to horizontal lines (provided the period is sufficiently long, vide p. 240!), which are  $e_o$  length units apart ( $e_o$  is the output "peak to peak" amplitude; the corresponding input amplitude, on the open circuit, i. e. without the object between  $a$  and  $b$ , is  $E$  units).

By some suitable timing device markings of time intervals are introduced on the cathode-ray trace. This can be done by modulating the cathode-ray beam with a standard frequency source, thereby "time dots" are obtained. An other simple method is to apply a timing sine frequency of a barely detectable amplitude superimposed only on the input square wave  $E'$  (cf. Fig. 5). These small "teeth" are later projected optically on the enlarged pencil drawings of the output wave to give the foot points of the y-ordinates described in the next section.

The cathode-ray screen trace is photographed with a good camera, preferably on 36 mm film. To each series of output

<sup>1</sup> A small condensor is used for the bridging between the timing oscillator and the oscillograph input.

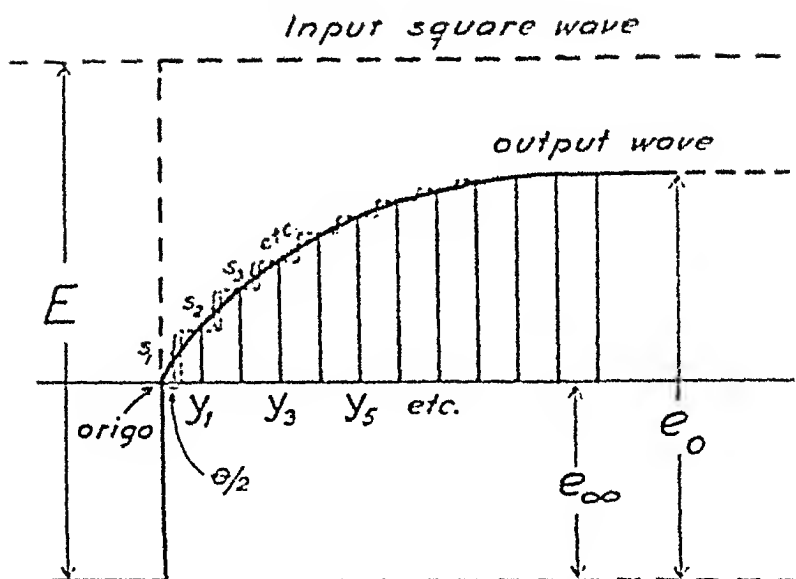


Fig. 6. Diagram illustrating the analysis of an output wave record.

Only the curved part above the "origo" line is subject to the harmonic analysis described in the text. The y-ordinates are used in the regular Fourier analysis, the "steps"  $s$  in the square wave analysis. — The lengths  $e_0$ ,  $e_\infty$  (and  $E$  belonging to the input wave) can directly yield D.C. and high frequency resistance when inserted in Eqs. 17 and 18. —  $\theta/2$  is the phase difference between the actual wave and its approximation i. e. the "stepped" wave (dotted).

exposures belongs one exposure of the open circuit amplitude  $E$  (with the time marking), all taken under identical amplification and time sweep conditions. The film record is later projected as an enlarged picture, which is copied on paper in lead pencil (with the standard timing scale added). These drawings, in a size of at least  $20 \times 30$  cm, are now used for the graphical analysis described in the next section.

## 2. A method of decomposing a transient output wave into sine wave components:

The following procedure is in the main based on a graphical analysis elaborated for television purposes by BEDFORD & FREDENDALL, who have also given a rather complete theory to which it may be referred (cf. also EMERY, p. 114). In the method below, however, an important theoretical correction has been included, which yields more accurate phase angles, and from a practical view point a convenient and simple vector addition apparatus has been constructed that is time saving.

On a photographically enlarged drawing or copy of the output wave the lengths of the steep rise  $e_\infty$  and the maximal ampli-



tudes  $E$  and  $e_0$  are measured. On the curved part ordinates  $y_1, y_2$ , etc. are drawn in the marks of the timing frequency with the origo as shown in Fig. 6 and their lengths measured. The greater number of ordinates taken, the better degree of accuracy will be obtained just as in the regular Fourier analysis which also starts

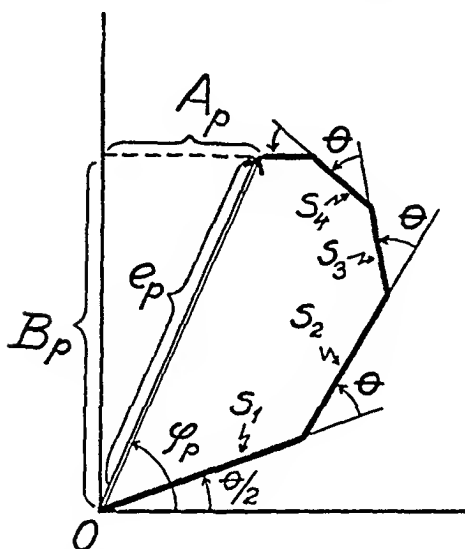


Fig. 7. Scheme of the vectorial addition.

The voltage "steps"  $s$  add up to a sum vector  $e_p$  with the polar coordinates  $e_p/\varphi_p$ , or rectangular coordinates  $A_p, B_p$ , which values are used for calculation of the impedance. The angle  $\Theta$  depends on the frequency to be analyzed.

to end according to Fig. 7, each  $s$  advanced an angle  $\Theta$  degrees in comparison with the previous  $s$ . It is important that  $s_1$  is started with an angle  $\Theta/2$  (see the figure).<sup>1</sup> The value of the vector angle  $\Theta$  is determined by the frequency  $N$  of the sine wave to be examined and the standard timing frequency  $f$  according to the expression

$$\Theta = \frac{N}{f} \cdot 360^\circ \dots \dots \dots (6)$$

<sup>1</sup> The reason for this procedure is that the "stepped" wave approximation lags the true response wave by one half a timing interval (cf. Fig. 6). This fact is mentioned by BEDFORD & FREDENDALL but was neglected by them because they were interested only in relative phase relations, which appear correct without the  $\Theta/2$  correction.

with erecting equidistant ordinates. Our experiences shows that at least 12 ordinates are required which must extend over the whole visibly curved part (it is not necessary to use ordinates under the practically horizontal part).

The following operation consists in tabulating the differences  $s_1 = (y_1 - 0)$ ,  $s_2 = (y_2 - y_1)$ ,  $s_3 = (y_3 - y_2)$  etc., which actually are the heights of the separate steps of a stepped wave that approximates to the original wave (as marked in the figure with the dotted curve.)

Vectorial addition of the steps " $s$ " is the next procedure, which takes place as follows: The lengths  $s_1, s_2, \dots$  are added consecutively end

(As the  $\Theta$ 's ought not to exceed about  $45^\circ$  the examination of higher  $N$ 's necessitates a finer time division, i. e. more  $y$ -ordinates must be drawn.)

The sum vector  $e_p$  obtained for a particular frequency is now representing a sinusoidal voltage vector, its magnitude being the

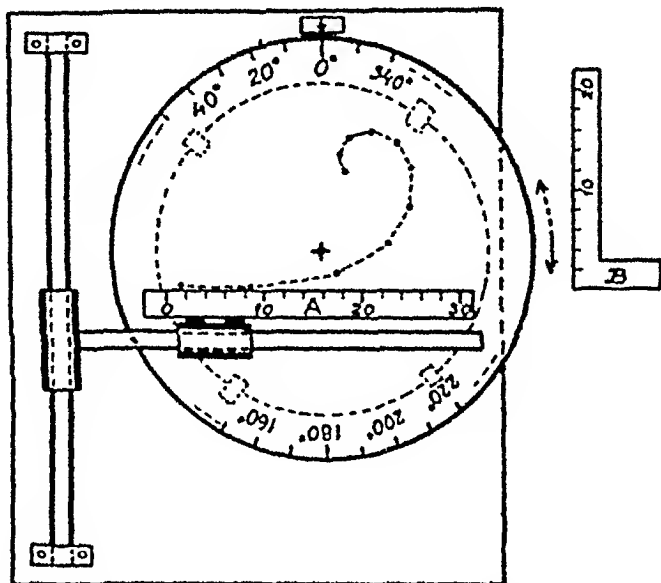


Fig. 8. Sketch of an apparatus for vectorial operations consisting of rotating disc calibrated in degrees and a scale (A) adjustable in the x-y-directions. B is a 90-degree scale. — A paper is mounted on the disc and, for instance, vectorial addition is carried out as sketched with the dot-dashed line. A suitable mechanical device can make convenient the rotation with constant angles.

peak to peak amplitude expressed in relative units and its angle  $\varphi_p$  being the phase shift relative to the input voltage.

The  $e_p$  of any frequency refers to the same constant, sinusoidal input amplitude  $E$  (this is a special feature of the Bedford-Fredendall procedure). If, however, a regular Fourier analysis was employed, one has to consider the fact that the harmonics of the square input wave vary in amplitude inversely as their frequencies according to Eq. 2.

For the purpose of a somewhat easier transformation of this voltage vector into an impedance vector it may be better to determine the former by its rectangular xy-coordinates, in the Fig. 7 indicated as  $A_p$  and  $B_p$ .

A simple apparatus for vector calculations: Already ROTHE (l. c) pointed out that the Fourier harmonic calculations would be simpli-

fied if *polar* coordinates were employed and recommended the use of "Windrosen" on polar graphical paper for the necessary vector additions. Recently BEDFORD & FREDENDALL also described the use of polar coordinate scales for their square wave analysis. The apparatus sketched in Fig. 8 is, however, more time saving and accurate than the polar paper scale procedures. This apparatus may be found useful also in the Fourier harmonic analysis [according to ROTHE (l. c.)] of non-asymptotic square wave responses of the type mentioned on p. 239.

On a base plate a plane, circular metal disc, ca 30 cm in diameter, is calibrated in degrees 0—360° and is movable with some friction around its center. By means of two "sledges" of metal tubings, mounted at right angles on metal rods, a millimeter ruler can be placed, always horizontal, in any position on the circular disc. On this disc a circular paper of a somewhat smaller diameter is fastened by aid of strips of adhesive tape.

The use is as follows: The vector lengths, for instance the steps "s", are drawn in sharp pencil along the ruler, the disc is then rotated the proper number of angle degrees (measured against a fixed pointer), the ruler is now moved to the last vector endpoint, a new vector is drawn etc. until all vectors are added (negative vectors are turned the opposite way). The length and angle of the sum vector are then easy to record. If desired, it is convenient to read instead the final xy-coordinates by the help of the scale "A" and a separate 90-degree scale "B".

3. *The transformations of voltage vectors into impedance vectors:* — Considering the principle equivalent scheme of Fig. 4, and employing vector notations throughout, the following conditions are valid for a particular frequency:

$$E = e_r + e_z \quad \dots \quad (7)$$

$$e_z = e_\infty + e_p \quad \dots \quad (8)$$

$$\frac{e_r}{e_z} = \frac{r}{Z} \quad \dots \quad (9)$$

The relations between the various voltage vectors is elucidated by the vector diagram Fig. 9.<sup>1</sup> In complex component form one can now write with the symbols of the diagram (*j* is the operator  $\sqrt{-1}$ )

$$e_z = A_1 + jB_p \text{ and } e_r = A_2 + j(-B_p)$$

These expressions inserted in Eq. (9) yield

$$Z = r \cdot \frac{e_z}{e_r} = r \cdot \frac{A_1 + jB_p}{A_2 - jB_p} \quad \dots \quad (10a)$$

<sup>1</sup> For the use of vector algebra applied to electrical circuit problems see references in footnote <sup>1</sup> on p. 237.

According to the rules of vector division one obtains

$$Z = r \cdot \frac{[A_1 A_2 + B_p(-B_p)] + j[A_2 B_p - A_1(-B_p)]}{A_2^2 + B_p^2} \dots (10b)$$

The impedance vector  $Z$  has accordingly in polar coordinates

$$\text{the magnitude } |Z| = r \cdot \sqrt{\frac{A_1^2 + B_p^2}{A_2^2 + B_p^2}} \dots (11)$$

$$\begin{aligned} \text{the phase angle } \varphi_Z &= \text{arctg} \frac{A_2 B_p - A_1(-B_p)}{A_1 A_2 + B_p(-B_p)} \\ &= \text{arctg} \frac{B_p E}{A_1 A_2 - B_p^2} \dots (12) \end{aligned}$$

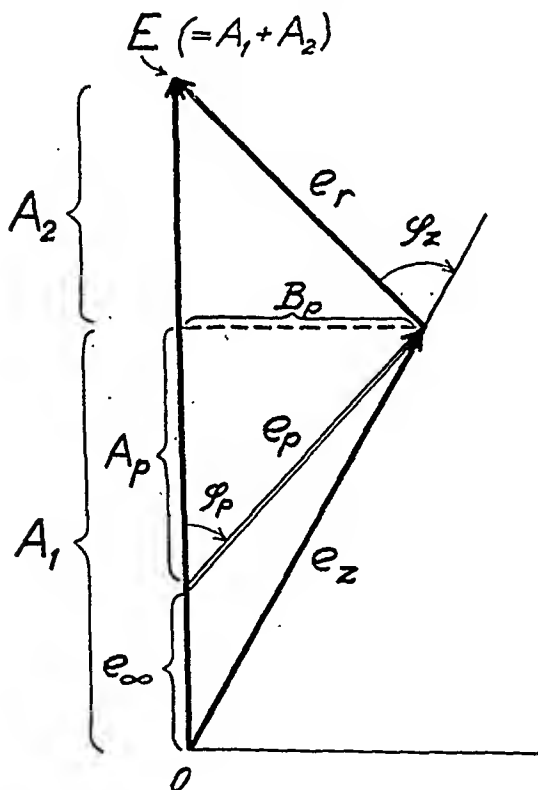


Fig. 9. Simple vector diagram of voltage relations in the equivalent circuit Fig. 4.  $E$  and  $e_\infty$  are relative voltages taken directly from the square wave records,  $A_p$ ,  $B_p$  result from square wave analysis (or Fourier analysis of the  $y$ 's of Fig. 6, when  $A_p = (n\pi/2)a_n$ ;  $B_p = (n\pi/2)b_n$ , cf. p. 239).  $e_r$ ,  $e_z$ ,  $e_p$  = voltage vectors;  $A_1$ ,  $A_2$  abbreviations in the formulas;  $\varphi_Z$  = phase angle of the impedance vector.

The impedance vector  $Z$  can be resolved, if desired, into its two components, *i. e.* the series resistance  $R$  and series reactance  $X$ , according to the following expressions derived from Eq. (10 b):

$$\text{the resistance } R = r \cdot \frac{A_1 A_2 - B_p^2}{A_2^2 + B_p^2} \quad \dots \dots \dots (13)$$

$$\text{the reactance } X = r \cdot \frac{B_p E}{A_2^2 + B_p^2} \quad \dots \dots \dots (14)$$

In these equations  $r$  is the total output resistance of the square wave generator, the symbols  $A_1$  and  $A_2$  are abbreviations composed of the quantities  $E$  and  $e_\infty$ , which were measured directly on the output wave or its projection (cf. p. 243 and Fig. 6), and of  $A_p$ , which was obtained, together with  $B_p$ , as results of the wave analysis described in the previous section:

$$A_1 = e_\infty + A_p \quad \dots \dots \dots (15)$$

$$A_2 = E - (e_\infty + A_p) \quad \dots \dots \dots (16)$$

The impedance vector  $Z$ , or its components  $R$  and  $X$ , are identical with corresponding quantities obtained with the common A.C. bridge methods and can now be plotted together with the  $Z$ -vectors (or  $R$  and  $X$  values) of other frequencies to the desired impedance locus diagram.

It should be noted that the impedance values for two particular frequencies, namely zero (the direct current value) and the frequency  $\infty$  are easily obtainable from the values  $E$ ,  $e_0$  and  $e_\infty$  which could be measured directly on the cathode-ray screen or the corresponding projection (cf. Fig. 3 or 6). Only a relative scale, for instance millimeters, is necessary. These special cases of Eq. 13 are

$$\text{Direct current resistance } R_0 = \frac{r \cdot e_0}{E - e_0} \quad \dots \dots \dots (17)$$

$$\text{High frequency resistance } R_\infty = \frac{r \cdot e_\infty}{E - e_\infty} \quad \dots \dots \dots (18)$$

### Some Applications.

In order to demonstrate the applicability of the square wave method for the determination of impedance quantities some examples will now be given.

1. A resistance-capacitance circuit composed of a resistor ( $104 \Omega$ ) and a radio condenser ( $8.4 \mu F$ ) with a shunt resistor ( $101 \Omega$ ) was employed as a "model" of the actual biological systems. The arrangement was the same as shown in Fig. 4. A square wave, unbalanced to earth of  $-20$  mV, and of 70 c.p.s. was impressed on the model circuit. The generator resistance  $r$  was  $209 \Omega$ . The

record on the 16 cm cathode-ray screen gave direct values on  $e_o$  and  $e_\infty$ , which inserted in Eqs. 17 and 18 yielded .

Direct current resistance  $R_o = 209 \Omega$  (theor. value 205)

High frequency resistance  $R_\infty = 109 \Omega$  ( " " 104)

Shunt resistance  $R_p (= R_o - R_\infty) = 100 \Omega$  ( " " 101)

The value of the capacitance was determined at the "characteristic frequency"<sup>1</sup> ( $N_c$ ) according to COLE's formula  $C_p = 1:2\pi N_c R_p$  and was found to be  $8.2 \mu F$  (theor. value 8.4). The positions of some experimental impedance values found by the square wave analysis (marked with crosses) can be compared with those mathematically calculated (marked with filled points) in the figure 10. The reason for the small displacement between the loci diagrams, caused by a somewhat too large  $R_\infty$ , is unknown, but is of a rather insignificant order from a practical point of view.

2. The effects of NaCl and KCl on frog skin shown in Table I constitute a good example of how changes in D.C. resistance ( $R_o$ ) and high frequency resistance ( $R_\infty$ ) can very rapidly follow some type of stimulating or injurious treatment of a living

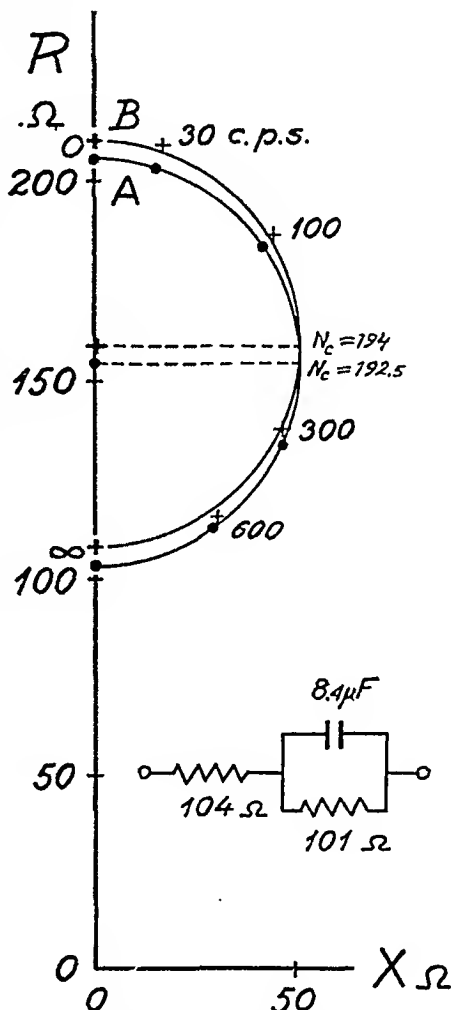


Fig. 10. Impedance locus diagrams of a biological equivalent circuit.

The filled points are theoretically calculated for a circuit of the type a-b of Fig. 4 where  $R_\infty = 104 \Omega$ ,  $R_p = 101 \Omega$  and  $C_p = 8.4 \mu F$ . The crosses belong to corresponding experimental results evaluated by square wave analysis. Actual records in Fig. 5.

<sup>1</sup> i. e. the frequency where the series reactance  $X$  is at maximum. It can be graphically interpolated from the actually measured frequencies. In this case when  $\phi = 90^\circ$  the  $C_p$  can also be calculated from any frequency point.

Table I. The effect on frog skin of NaCl and KCl on the external side.

*Ringer-Gray solution in the inside (i)-chamber.*

Time	Treatment	$R_o$ $\Omega$	$R_\infty$ $\Omega$	$R_p$ $\Omega$	$N_c$ c. p. s.	(o)-(i) mV
0 <sup>h</sup> 00 <sup>m</sup>	Preparation finished . .	—	—	—	—	—
01 <sup>m</sup>	N/10 NaCl outside . . .	535	118	417	—	- 25
1 <sup>h</sup> 36 <sup>m</sup>	— , — . . .	1,700	123	1,577	(30)	- 53
50 <sup>m</sup>	— , — . . .	1,495	120	1,375	—	- 54
2 <sup>h</sup> 08 <sup>m</sup>	— , — . . .	1,560	122	1,348	43	- 55
11 <sup>m</sup>	N/10 KCl outside . .	—	—	—	—	—
12 <sup>m</sup>	— , — . . .	2,610	116	2,494	35	- 21
40 <sup>m</sup>	— , — . . .	2,090	116	1,974	32	- 21
3 <sup>h</sup> 16 <sup>m</sup>	— , — . . .	1,870	111	1,579	—	- 29
33 <sup>m</sup>	— , — . . .	1,870	112	1,758	30	- 30
35 <sup>m</sup>	N/10 NaCl outside . . .	—	—	—	—	—
37 <sup>m</sup>	— , — . . .	1,215	119	1,096	62	- 72
42 <sup>m</sup>	— , — . . .	1,195	120	1,075	—	- 76
4 <sup>h</sup> 39 <sup>m</sup>	— , — . . .	1,490	125	1,365	65	- 70
Control	Filter paper, N/10 NaCl (o)	101	90	11	—	+ 4
,	, , N/10 KCl (o)	92	79	13	—	+ 0.3

The polarization angle  $\varphi_p$  was always between 85°—90°.<sup>1</sup>

biological object. The experimental set up was analogous to the one described by TEORELL & WERSÄLL. With the square wave method now used it was easy to evaluate  $R_o$  and  $R_\infty$  from measurements directly on the cathode-ray screen as described above (p. 248). These values are the ones recorded in the table. The characteristic frequencies  $N_c$  were determined from separate film records analyzed according to the procedure described in this paper. The details of the result of this analysis will, however, be published in full in a later publication. Here we will only point out that the exchange of a N/10 NaCl solution against N/10 KCl caused a marked increase in particular of the parallel resistance  $R_p$ , the  $N_c$  changed but little (decreased), whereas the high frequency resistance  $R_\infty$  remained practically constant. It is

<sup>1</sup> I. e. almost perfect semicircular loci were obtained for the impedance vectors resulting from the square wave analysis (the definition of  $\varphi_p$  is evident from Fig. 1).

Table II. The effect of D. C. stimulation on frog skin.

Ringer-Gray solution in (i)-chamber, N/10 NaCl in the (o)-chamber.

Time	Treatment	$R_o$ $\Omega$	$R_\infty$ $\Omega$	$R_p$ $\Omega$	$N_c$ c. p. s	(o)-(i) mV
0 <sup>h</sup> 00 <sup>m</sup>	Preparation finished . .	—	—	—	—	—
57 <sup>m</sup>	— , — . .	905	139	666	83	— 32
2 <sup>h</sup> 44 <sup>m</sup>	— , — . .	694	169	526	—	— 23
3 <sup>h</sup> 13 <sup>m</sup>	Before stimulation . . .	707	155	552	86	— 28
13.5 <sup>m</sup>	1 V =, (i) ← (o), 0.5 <sup>m</sup> .	348	156	192	590	—
14 <sup>m</sup>	After stimulation . . .	443	163	280	—	— 7 to — 28
15 <sup>m</sup>	— , — . . .	522	163	359	140	— 38
20 <sup>m</sup>	— , — . . .	562	156	442	120	— 33
—	— , — . . .	—	—	—	—	—
4 <sup>h</sup> 48 <sup>m</sup>	Before stimulation . . .	777	159	618	62	— 37
50 <sup>m</sup>	1 V =, (i) → (o), 0.5 <sup>m</sup> .	246	150	96	410	—
51 <sup>m</sup>	After stimulation . . .	348	154	194	—	— 2 to — 7
52 <sup>m</sup>	— , — . . .	413	156	257	175	— 10
55 <sup>m</sup>	— , — . . .	545	155	390	111	— 18

The 'polarization angle'  $\phi_p$  was always between 85°—90°.¹

also worth noticing that the "membrane potential" is always depressed simultaneously with the  $R_p$  increase caused by the KCl addition.¹

3. *The effect of D.C. stimulation on frog skin* is another case, where the square wave method is especially indispensable, because impedance changes here take place very rapidly under and after the D.C. shock. As an example, extracted from a study to be published later, we here refer to Table II, which demonstrates that the current flow exerts a great influence on the parallel resistance (as well as on the membrane potential). In this case also great changes in  $N_c$  were observed, which were roughly inversely proportional to the  $R_p$  changes. (Incidentally, this would signify, according to the COLE formula above, that the "skin capacitance" remained rather unchanged in spite of the stimula-

¹ Related effects of NaCl and KCl on the frog skin potentials have been independently studied by K. H. MEYER & BERNFELD who also will discuss the possible implications in a forthcoming issue of the J. Gen. Physiol. (according to a personal communication from Prof. K. H. MEYER, Genève).



tion, in analogy with the observations on stimulation of *Nitella* or *Loligo* reported by COLE et coll. (1938 and 1940).) Another remarkable effect was that the more or less complete annihilation of the membrane potential in connection with the electrical "shock" was followed by a recovery, which was considerably slower after stimulation with an "outwardly" directed current than after a current flow in the opposite direction. This was true both as regards the parallel resistance  $R_p$  and the skin potential.

### Concluding Remarks.

The exact significance of the parameters in the GILDEMEISTER-LULLIES-COLE method of characterizing cell or tissue impedance is far from clear. Hence, great caution is necessary in all attempts to interpret these impedance quantities in terms of cell or tissue permeability. Nevertheless it is a fact that these A.C. impedance quantities ( $R_p$ ,  $R_\infty$ ,  $N_c$  and  $\varphi_p$ ) can be employed on biological objects as useful measures on alterations, caused by stimulation or injury. In this connection one may mention that the use of the inverse impedance value, i. e. the "admittance", often is a more rational and convenient concept to employ in biological studies.

It might be questioned, however, whether the "symbolical" impedance (or admittance) locus method in many cases could not be with advantage replaced by analysis of the relations between applied direct current transients and time as suggested by LULLIES. Actually, the square wave response shows both the transient and steady state D.C. conditions. Thus it is evident that also in such types of "polarization" studies the use of repeating square waves is of great value. Such a method and the A.C. impedance method are, however, not different, they are only two different modes of approach to a common problem, namely the behaviour of ions and charged material in living tissues.

### Summary.

As a whole the present paper deals with a method, based on the use of square shaped electrical waves, that permits the registration of even very transient electrical impedance changes in biological objects. Directly it is possible to record a) the "transient"

and steady state D.C. resistance, b) the "high frequency" A.C. resistance, and also, after performing a procedure of "square wave analysis", c) to evaluate the impedance values for *any* intermediate A.C. frequency. Thus the method allows the construction of "impedance locus diagrams" (according to GILDEMEISTER-LULLIES-COLE) of phenomena of short duration, as, for instance, the impedance changes in living objects in connection with electrical stimuli shocks. The paper is disposed as follows:

1. In the introduction of the paper the theoretical relations are discussed which exist between the responses on sinusoidal alternating currents, square wave alternating currents and "rechteckige Stromstöße" (according to LULLIES).

2. A practical procedure of harmonic analysis is then described, which enables the transformation of the square wave response into impedances to sinusoidal currents.

3. As examples of possible applications are described: a) the response of a "model" circuit, b) the effects of NaCl and KCl solutions on frog skin, c) the effects of D.C. stimulation on frog skin.

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## Pain Conduction in the Phrenic Nerve.

By

B. GERNANDT.

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That stimulation of the central part of the diaphragm in man elicits pain referred to the shoulder region is a long known phenomenon which has been described among others by LUSCHKA (1863), FELIX (1922), MORLEY (1931), WOOLHARD, ROBERTS and CARMICHAEL (1932) and HINSEY and PHILLIPS (1940).

In animal experiments POLLOCK and DAVIS (1935) explained this pain as being produced by visceromotor reflexes which elicited vasomotor and chemical processes in the shoulder region. The impulses were considered to be conducted by sympathetic fibres which in their turn excited somatic receptors of the shoulder region thus eliciting the referred pain. HINSEY and PHILLIPS in their animal experiments found, however, that the nociceptive reaction elicited by faradic stimulation of the diaphragm was entirely dependent upon the impulse traffic in the afferent somatic fibres of the phrenic nerve and that these reactions were not elicited by impulses conducted in afferent fibres of the vagus, the intercostal nerves or by impulses in any sympathetic efferent fibres.

GREEN (1935) showed that stimulation of the central end of the severed phrenic nerve produced a dilatation of the coronary vessels. THORNTON (1937) found that at least one afferent pathway of the broncho-dilatory reflex mechanism is situated in the phrenic nerve, the efferent path running in the vagus. Further, LITTLE and McSWINEY (1938) found that the phrenic nerve contains an afferent pathway for the pain impulses elicited by stimulating the diaphragm. In experiments upon the cat they used the

pupillo-dilatory reflex as an index of the existence of afferent impulses. The afferent pathways reached the spinal cord via the dorsal roots of the fifth and sixth cervical segments.

In a histological investigation by HINSEY, HARE and PHILLIPS (1939) these authors could show by degeneration experiments on the somato-motor and sympathetic fibres of the cat's phrenic nerve that this nerve contains myelinated afferent fibres of various sizes as well as unmyelinated afferent fibres. The afferent fibres amounted to about ten per cent of the total number of the myelinated fibres of this nerve, a ratio which is low in comparison to the motor branches of the femoral nerve, where thirty to forty per cent of the myelinated fibres are afferent.

The purpose of the present investigation was to record the afferent impulses elicited by noxious stimulation of the diaphragm. These experiments form part of a general survey in progress here upon the impulse traffic in afferent fibres, and yield somewhat more detailed information concerning the afferent fibres involved in "phrenic pain".

### Methods.

Cats have mainly been used but one experiment was done upon a dog. The animals were anaesthetized by 0.05 to 0.07 g chloralose per kg body-weight, which was injected intravenously in a 1 per cent solution.

The phrenic nerve was exposed in the neck and was dissected free in the caudal direction. The nerve was then severed as centrally as possible. At the moment of severance the animal always reacted with a violent twitch. In order to obtain as high a signal-to-noise ratio as possible, the sheath was generally pulled off the nerve. The nerve was frequently irrigated with body-warm Ringer solution in order to avoid drying.

The abdominal cavity was then opened in the midline in the region of the xiphoid process in the caudal direction so that it was easy to reach the abdominal surface of the diaphragm.

The recording of the action potentials was made by means of a capacity-resistance coupled amplifier and a double ray cathode oscillograph previously described by ZOTTERMAN (1936). The potentials were lead off by means of AgCl-electrodes of the conventional type.

### Results.

*The afferent inflow in the phrenic nerve:* With the leads at the central end of the phrenic nerve there occurred at each inspiration a massive volley of rapidly conducted impulses, whilst other-

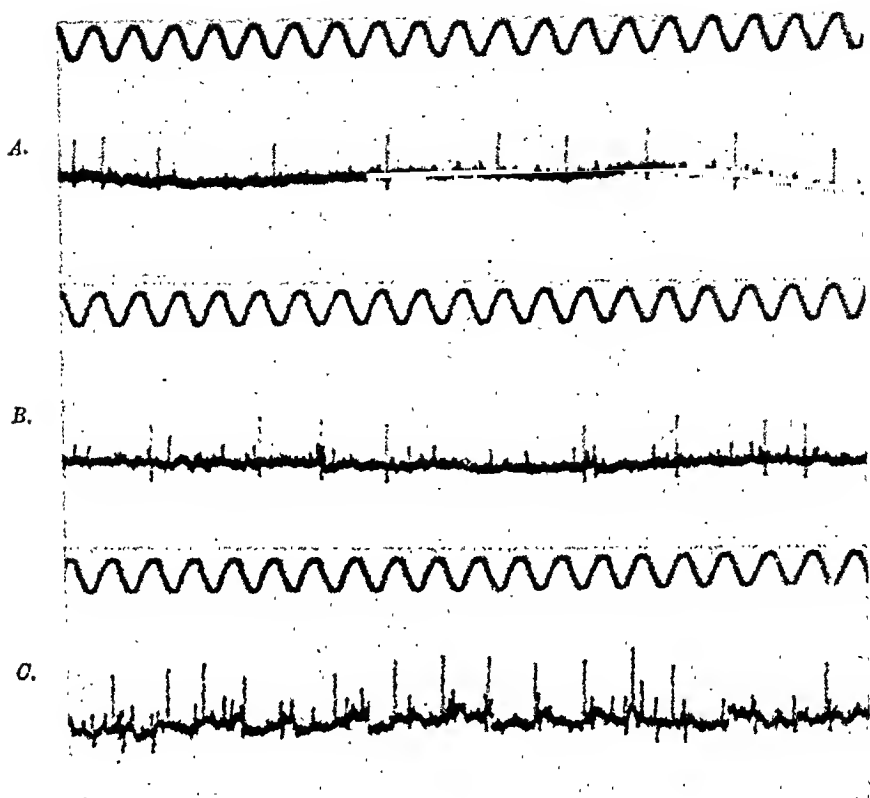


Fig. 1. Cat, chloralose. Action potentials from the peripheral end of the phrenic nerve. *A.* Control. The electro-neurogram is taken during expiration. *B.* Brushing the abdominal surface of the diaphragm with 0.5 N acetic acid. *C.* Pinching of the diaphragm. Note the occurrence of the small, slowly conducted  $\delta$ -potentials.

wise there was only very slight activity. These rapid impulses are obviously produced by fairly large myelinated fibres belonging to the  $\alpha$ - and  $\beta$ -group of the class A fibres.

Pinching the diaphragm with forceps or brushing its abdominal surface with 0.5 N acetic acid led to twitches, tail movements, pupillar dilatation and a moderate rise of the arterial blood pressure. Simultaneous recording of the action potentials from the phrenic nerve of the cat revealed an appearance of slowly conducted small spike potentials (fig. 1), which seem to derive from fairly thin fibres of the  $\delta$ -group, the system of the fastest pain fibres. The effects of very strictly localized pinching in the right or left central part of the diaphragm showed that the regions of innervation of the two phrenic nerves in any case

A.



B.

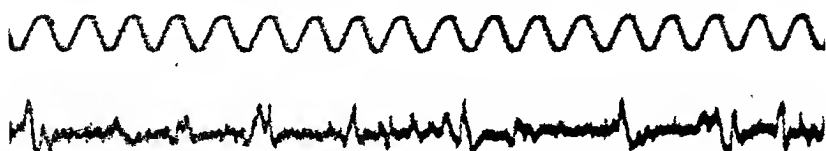


Fig. 2. Dog, chloralose. Action potentials from the peripheral end of the right phrenic nerve. *A.* Control. The electro-neurogram is taken during expiration. *B.* After infiltration of the central and right part of the diaphragm with 0.75 cc of a 6 per cent NaCl solution. Note the waves built up by very slowly conducted C-potentials.

were rather well separated from each other. It looked as though there was not very much of overlapping of the pain fibres.

In the experiment on the dog the diaphragm was infiltrated by 0.75 ml of a 6 per cent NaCl solution. This produced waves of very slow impulses (fig. 2). These waves should probably be looked upon as produced by the summation of a number of single C-spikes. As the ratio between non-myelinated and myelinated fibres in the cat's phrenic nerve is rather high compared with the motor branches of its femoral nerve, noxious stimuli of the central part of the diaphragm were also expected to produce C-potentials but such potentials were never observed in the experiments on the cat.

The findings thus support the opinion (ZOTTERMAN 1936, 1939) that nociceptive reactions are not elicited by the more rapidly conducted fibres of the  $\alpha$ - and  $\beta$ -groups but that the fibres which transmit pain are to be found among  $\delta$ - and C-fibres (ZOTTERMAN 1933, CLARK, HUGHES and GASSER 1935, LEWIS and POCHIN 1938, GERNANDT and ZOTTERMAN 1946).

Light touch or a faint puff of air over the diaphragm which latter does not produce any deformation of the surface does not

elicit any impulses. Nor were there any impulses from the application of cold ( $+4^{\circ}\text{C}$ ) or moderate heat ( $+40^{\circ}\text{C}$ ). Stronger heat ( $+45^{\circ}\text{C}$ ), however, produced in the cat a volley of  $\delta$ -potentials.

RAUBER has described the presence of Pacinian corpuscles in man along the endings of the phrenic nerve in the pericardium and pleura. Such formations could not be found in the cat or the dog by macroscopic examination. Along the mesenteric nerves of the cat there is an abundance of these corpuscles which are easily observed by the naked eye. These corpuscles were found by GERNANDT and ZOTTERMAN (1946) to be very sensitive to the slightest mechanical stimulus. Thus the slightest puff over the mesentery elicited a massive volley of large spikes. In case there really existed any Pacinian corpuscles in the pericardium of the cat with their nerve fibres running in the phrenic nerve one should thus expect a volley of large spike potentials at each heart beat. This, however, was not the case. Other mechanical stimuli such as puffing or gentle brushing applied to the pericardium or to the pleura did not produce any large spikes from the phrenic nerve.

### Summary.

The application of noxious stimuli to the diaphragm (pinching, heat above  $40^{\circ}\text{C}$ , hypertonic NaCl-solutions and acetic acid) produces in the phrenic nerve of the cat  $\delta$ -potentials whilst in the dog both  $\delta$ - and C-potentials appear.

Each inspiration produces a volley of rapidly conducted impulses in the phrenic nerve. These impulses are obviously produced by  $\alpha$ - and  $\beta$ -fibres. Large spikes are not produced by a noxious stimulus such as acetic acid applied on the abdominal surface of the diaphragm. It is thus concluded that pain is transmitted in the phrenic nerve of the animals investigated by  $\delta$ - and C-fibres only.

Pacinian corpuscles have not been observed in the pericardium or in the pleura of the cat or the dog. The action potential records from the peripheral end of the phrenic nerve have not revealed the appearance of any spikes which could be regarded as arising from such corpuscles.

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## The Effect of Excitation on Nerve Permeability.

By

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The effect of muscular exercise on the permeability of muscular tissue to phosphates (BOLLMAN and FLOCK 1943, HEVESY and REBBE 1946) and potassium (HAHN and HEVESY 1941, NOONAN, FENN and HAEGE 1941) has previously been investigated by using  $^{32}\text{P}$  and  $^{42}\text{K}$  as indicators. Phosphate-permeability was found to be influenced only to a minor extent by muscular exercise, while the amount of potassium was found to be increased 3 to 4 times as the result of intense muscular work.

The present communication gives the results of experiments in which the effect of excitation on the permeability of the sciatic nerve for phosphate, sodium, potassium and bromide was investigated.

Radioactive isotopes were used as indicators.<sup>1</sup>

### Experimental Procedure.

Cats weighing about 2 kg were used. Chloralose, 5–6 ml of an 1 per cent solution per kg, was injected into a brachial vein under ether anaesthesia, except in the first case (Table 1), where it was injected into the femoral vein on the stimulated side. The sciatic nerves were exposed near the spinal cord on each side and crushed with a forceps. Stimulation was effected by means of a thyatron stimulator giving condensor shocks at a rate of about 50 per sec. and at a strength producing about maximal motor reactions. In one experiment (sodium No 5) the animal was curarised before stimulation in order to avoid muscular movements.

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<sup>1</sup> Our thanks are due to Prof. NIELS BOHR and to Prof. M. SIEGBAHN for the radio-active preparations kindly put at our disposal.

The radioactive salts, dissolved in a few ml of distilled water, was injected intravenously through the cannula used for the anaesthetic. In the majority of cases the stimulation was carried on for 5 minutes and the active preparation injected at the end of the second minute of stimulation. Immediately after the stimulation a sample of heparinized blood was taken by heart puncture and centrifugated, the plasma being used for determination of the activity. The animal was killed by bleeding and the hind legs washed free of blood by perfusion with Ringer's solution through a cannula in the lower part of the aorta. When the outflowing blood was macroscopically free from blood corpuscles the sciatic nerves on both sides were carefully dissected out and worked up.

## Results.

### Phosphate Permeability.

In Table 1 are given the weights of the fresh and dry nerve, the percentage of dry substance, the total phosphorus content of the nerve, the phosphorus content per g fresh weight, the ratio of the activity per g. dry stimulated and non-stimulated sciatic nerve, the ratio of the activities of 1  $\mu$ g phosphorus extracted from the stimulated and non-stimulated nerve and finally the percentage activity of 1  $\mu$ g total nerve phosphorus relative to that of 1  $\mu$ g inorganic phosphorus.

Table 1.

*Weight of cat 2.4 kg. Left nerve stimulated for 6 min. At the end of the first minute 0.75 millicurie  $^{32}\text{P}$  injected intravenously. Inorganic plasma  $\text{P} = 6.10 \text{ mg p. c.}$*

	Fresh weight of sciatic nerve in mg	Dry weight in mg	Percentage dry substance	P content in $\mu\text{g}$	mg P per g fresh nerve	Ratio of activities of 1 g dry irritated and 1 g non-irritated nerve	Ratio of activities of 1 $\mu\text{g}$ P extracted from irritated and non-irritated nerves	Percentage activity of 1 $\mu\text{g}$ nerve P to 1 $\mu\text{g}$ inorganic plasma P
Stimulated . .	415.7	135.4	32.6	2040	4.92	1.18	1.10	0.237
Non-stimulated	476.2	154.1	32.4	2110	4.04			0.216

It will be seen from the figures in Tables 1 to 4 that the irritated nerve in each case took up more  $^{32}\text{P}$  than the non-irritated nerve, the ratio varying between 1.18 and 2.46. For the ratio of the quotients  $\frac{^{32}\text{P}}{^{31}\text{P}}$  for the irritated and non-irritated nerves figures varying between 1.10 and 1.98 were obtained.

Table 2.

Weight of cat 2.95 kg. Left nerve stimulated for 5 min. At the end of the second minute 0.3 millicurie  $^{32}\text{P}$  injected intravenously. Inorganic plasma  $\text{P} = 4.42 \text{ mg p. c.}$

	Fresh weight of sciatic nerve in mg	Dry weight in mg	Percentage dry substance	P content in $\mu\text{g}$	mg P per g fresh nerve	Ratio of activities of 1 g dry stimulated and 1 g non-stimulated nerve	Ratio of activities of 1 $\mu\text{g}$ P extracted from stimulated and non-stimulated nerves	Percentage activity of 1 $\mu\text{g}$ nerve P to 1 $\mu\text{g}$ inorganic plasma P
Stimulated . .	312.1	104.2	33.4	1320	4.18	2.46	1.98	0.080
Non-stimulated	394.6	127.4	32.0	1324	3.36			0.040

Table 3.

Weight of cat 2.4 kg. Left nerve stimulated for 5 min. At the end of the second minute 0.4 millicurie  $^{32}\text{P}$  injected intravenously.

	Fresh weight in mg	Dry weight in mg	Percentage dry substance	P content in $\mu\text{g}$	mg P per g fresh nerve	Ratio of activities of 1 g dry stimulated and 1 g non-stimulated nerve	Ratio of activities of 1 $\mu\text{g}$ P extracted from stimulated and non-stimulated nerves	Percentage activity of 1 $\mu\text{g}$ nerve P to 1 $\mu\text{g}$ inorganic plasma P
Stimulated . .	—	117	—	1474			1.50	0.109
Non-stimulated	—	113	—	1350				0.075

Table 4.

Weight of cat 1.8 kg. Left nerve stimulated for 5 min. At the end of the second minute 0.4 millicurie  $^{32}\text{P}$  injected intravenously. During the last minute intermittent stimulation (2.5 sec. irritation and 2.5 sec. rest).

	Fresh weight of sciatic nerve in mg	Dry weight in mg	Percentage dry substance	P content in $\mu\text{g}$	mg P per g fresh nerve	Ratio of activities of 1 g dry stimulated and 1 g non-stimulated nerve	Ratio of activities of 1 $\mu\text{g}$ P extracted from stimulated and non-stimulated nerves	Percentage activity of 1 $\mu\text{g}$ nerve P to 1 $\mu\text{g}$ inorganic plasma P
Stimulated . .	—	113	—	1048	—		1.35	0.137
Non-stimulated	—	101	—	1074	—			0.089

If we wish to know the percentage of P present in the nerve tissue (cf. above) that was taken up in the course of the experiment, we must compare the specific activity of the nerve P with the average value for the inorganic P of the plasma. This magnitude does not necessarily correspond to the amount that has penetrated from the plasma into the tissue during the experiment, as it is conceivable that a part of the P migrating from the plasma into the tissue cells has found its way back again. Considering that the amount of plasma P located in the nerve tissue constitutes only a small percentage of the total P content of the tissue and in view of the rapid participation of intruded phosphate in phosphorylation processes, we may suppose the amount of labelled P (plasma P) located in the nerve tissue to be practically identical with that penetrating from the plasma into the nerve tissue during the experiment. Assuming that the specific activity of the plasma inorganic P of the cat declines after intravenous injection at the same rate as in a rabbit of the same weight, the average specific activity of the inorganic P in the plasma works out in a 3-minute experiment to be about 3 times that of the experimentally determined amount. For the amount of P penetrating during 3 minutes into the resting nerve tissue (cf. Tables 3 and 4)

we hence obtain the values  $\frac{0.08}{3}$  and  $\frac{0.09}{3}$  as percentage activity of nerve P to plasma P. In the experiment recorded in Table 1 the approximate value  $\frac{0.22}{5}$  is obtained. As discussed above the average specific activity of the plasma P is higher than its end value which was determined experimentally. To account for this decrease we have in the experiment taking 5 minutes to divide the percentage activity of nerve P to plasma P with the figure 6 which should give an approximately correct value.

The amount of plasma P passing into 1 g fresh resting nerve tissue in the course of 1 minute proves on the average to be 0.01 per cent of the total P of the nerve tissue, thus about 0.4  $\mu$ g.

The amount of phosphorus penetrating in 1 minute into the nerve tissue is smaller than the corresponding amount penetrating into the muscle cells. KALCKAR and his associates (1944) estimate that, in the course of 1 minute, 1  $\mu$ g phosphorus penetrates into the cells of 1 g fresh muscle tissue of the rat. A similar figure is reported by HEVESY and H. v. EULER (1942).

**Sodium Permeability.**

In these experiments labelled sodium with an activity of about 0.5 millicurie was injected into the circulation. The cat was killed 5 minutes after the injection. In each experiment, as seen in Table 5, the irritated nerve was found to take up more  $^{24}\text{Na}$  than the resting nerve.

Table 5.  
*Uptake of  $^{24}\text{Na}$  by sciatic nerve.*

Weight of cat in kg	Ratio of uptakes by irritated and resting nerves	Activity of 1 mg dry nerve as percentage of activity of 1 mg dry plasma	
		irritated	resting
I. . . . . 3.5	1.40	0.77	0.55
II. . . . . 1.85	1.56	1.38	0.81
III. . . . . 1.9	1.06	2.67	2.48
IV. . . . . 2.2	1.98	4.80	2.42
V. . . . . 1.85	1.32	1.79	1.36

Assuming 1 mg dry plasma to contain 40  $\mu\text{g}$  sodium and the mean activity of sodium during the 5-minute experiment to amount to 3 times that of the activity measured at the end of the experiment, 1 mg dry resting nerve takes up on an average 0.2  $\mu\text{g}$  sodium per minute. In view of the very rapid change in the  $^{24}\text{Na}$  content of plasma which follows intravenous injection, this figure represents only a rough estimate of the amount of sodium taken up by the nerve.

**Potassium Permeability.**

In these experiments labelled potassium chloride having a specific activity of 0.3 millicurie was injected. The injection took 1 minute, the cat was killed 2 minutes later. The results obtained are seen in Table 6.

Table 6.  
*Uptake of  $^{42}\text{K}$  by sciatic nerve.*

Weight of cat in kg	Ratio of uptakes by irritated and resting nerves	Activity of 1 mg dry nerve as percentage of activity of 1 mg dry plasma	
		irritated	resting
I. . . . . 3.0	2.29	6.62	2.88
II. . . . . 1.9	1.51	8.60	5.83

By a similar consideration as put forward in the case of sodium we arrive to the conclusion that in the course of 1 min. about  $0.02 \mu\text{g}$  potassium penetrates in 1 mg dry nerve tissue or  $6 \mu\text{g}$  in 1 g fresh tissue.

It is of interest to compare this figure with the data recently obtained by HODGKIN and HUXLEY (1946) when determining the number of moles of potassium which leak through 1 sq.cm of membrane of the axons from *Carcinus maenas* in one impulse ( $1.7 \cdot 10^{-12}$ ) and the amount of potassium reabsorbed during the period of recovery. When the external potassium concentration is increased threefold,  $3 \cdot 10^{-10} \text{ g mol cm}^{-2} \text{ sec}^{-1}$  or  $0.7 \mu\text{g}$  potassium per minute were found to be reabsorbed.

### Bromine Permeability.

In these experiments 30 mg bromine as sodium bromide, with an activity of 0.2 millicurie was injected.

Table 7.  
*Uptake of  $^{82}\text{Br}$  by sciatic nerve.*

Weight of cat in kg	Ratio of uptakes by irritated and resting nerves	Activity of 1 mg dry nerve as percentage of activity of 1 mg dry plasma	
		irritated	resting
I. . . . . 1.98	1.34	3.4	2.9
II. . . . . 2.2	1.72	4.0	2.3

Assuming, as in the case of  $^{24}\text{Na}$ , that the average plasma activity during the experiment amounts to about 1/3 of the determined end activity, 1 mg dry resting nerve took up  $0.01 \mu\text{g}$  bromine. Similar amounts of radiobromine were found to be taken up by the basal ganglia, cerebral cortex and the medulla oblongata of the cat.

In no single case did the stimulated nerve fail to show an enhanced uptake of the ion investigated. When we compute the mean of all determinations (13 cases) of the ratio of uptake of various ions by the stimulated and the resting nerves, the value  $1.55 \pm 0.10$  is obtained. The uptake by the stimulated nerve was also found to be enhanced when the cat was curarized before stimulation in order to avoid muscular movement.

### Summary.

The effect of stimulation on the amounts of phosphate, sodium, potassium and bromide taken up by the sciatic nerve of the cat was investigated with the aid of radioactive isotopes as indicators. Stimulation was effected by condensor shocks at a rate of 50 per sec. giving maximal motor reactions.

In each case investigated, including the curarized animal, the stimulated nerve was found to take up more labelled ions than the resting nerve, the mean ratio of the uptakes being  $1.55 \pm 0.10$ .

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## The Regulation of Respiration during Muscular Work.

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It is a well-known fact that respiration during muscular work is, on the whole, adapted to the increased oxygen consumption; our knowledge of the mechanism, responsible for this adaptation is, however, still very incomplete. During light work, an increased alveolar carbon dioxide tension is sometimes found, but this is by no means a regular occurrence, and during heavy work the alveolar carbon dioxide tension is often decreased. It is therefore certain that the carbon dioxide tension of the blood alone cannot be the regulating factor. Nor can the hydrogen ion concentration of the arterial blood per se be decisive as was once supposed. Though several investigators have demonstrated an increased  $cH$  of the blood during light work, during heavy work an alteration in the reverse direction has also often been found. To explain the conflicting observations two different hypotheses have been advanced. According to one, first proposed by WINTERSTEIN (1921) and particularly upheld by GESELL and his school, the deciding factor is the  $cH$  of the respiratory centre itself which is not necessarily determined by the  $cH$  of the arterial blood, and sometimes even may vary in the opposite direction. The other view expounded by Danish investigators (LINDHARD 1911, 1933, NIELSEN 1936) maintains that the excitability of the respiratory centre is variable. Direct experiments by NIELSEN have shown that a certain increase in alveolar carbon dioxide tension during muscular work causes a greater increase of ventilation than during rest, and that this effect is to some extent related to the amount of work performed. NIELSEN considers this as due to an increased

excitability of the respiratory centre during work. It must be remembered, however, that during muscular work considerable alterations with regard to the distribution of ions between the cells and the blood may occur, so that the carbon dioxide tension of the arterial blood need not exactly reflect conditions within the cells of the respiratory centre. There is also the possibility of varying influences from the chemoreceptors, which have not been considered by NIELSEN.

In order to explain the increased response of the respiratory centre during muscular work at a given alveolar carbon dioxide tension, it has been assumed that during muscular work products appear which have a direct or indirect effect on the centre, or that the centre might be influenced by centrifugal cortical impulses simultaneously with the impulses to the muscles, or by afferent impulses from the acting muscles themselves. In man, ASMUSSEN, CHRISTENSEN and NIELSEN (1943) performed comparative experiments on oxygen consumption and ventilation during light and moderate work on the bicycle ergometer, under normal conditions and after the circulation in the legs had been occluded by inflation of rubber air cushions encircling the legs, under a pressure of 250—300 mm Hg. When the circulation had been cut off, oxygen consumption during work was about 20—50 percent lower than during free circulation, but total ventilation rose to practically the same levels in the two series. The authors conclude that the increased response of respiration to the stimulation by carbon dioxide during muscular work cannot be due to substances set free in the active muscles, but must be of nervous origin. ASMUSSEN, NIELSEN and WIETH-PEDERSEN (1943) have then made a comparison between the results during voluntary work and work induced by electrical stimulation by an apparatus simulating the normal nerve impulses. In a healthy subject, ventilation increased similarly in both series, in proportion to the oxygen consumption. From this, it was concluded that the increased excitability of the respiratory centre for carbon dioxide cannot be due to irradiation of central impulses but must be of reflex nature. In support of this view are the observations of HARRISON, CALHOUN and HARRISON (1932) in dogs, the hind-legs of which were connected to the body by only the sciatic nerves and the blocked vessels. Passive movements of the hind-legs still led to a small increase in ventilation. In a patient suffering from tabes dorsalis and with all the usual kinesthetic nervous impulses

abolished ASMUSSEN, NIELSEN and WIETH-PEDERSEN observed the normal increase of ventilation during voluntary as well as electrically induced work. The authors therefore believe that the reflexes which control ventilation during muscular work must employ other sensory paths than the posterior columns in the spinal cord.

COMROE and SCHMIDT (1943), in man, also found an increased ventilation when the work was performed with one arm, the circulation of which was cut off. The rise in ventilation (28 percent of the resting value) was on an average twice as great as when the circulation was free. The probable reason for this difference is, according to COMROE and SCHMIDT, the sensation of pain. This view was strengthened by the fact that respiration became normal again when the work was stopped and the pain subsided, in spite of the blood flow still being cut off. Since HENDERSON's (1910) and MEYER's (1914) papers, the stimulating effect on respiration from sensory stimulation, especially pain, is well known. Whether this factor can have been of decisive influence in the experiments by ASMUSSEN, CHRISTENSEN and NIELSEN quoted above, must be taken into consideration. These authors point out that psychological influence and ischemic pain increase as the experiment goes on, but maintain that this factor may be almost eliminated after some training. No definite proof has been given that such is the case, however, and it will probably be difficult to produce it. The conclusion drawn from the experiments is therefore open to doubt.

COMROE and SCHMIDT also stimulated electrically the peripheral end of the cut anterior nerve roots in anesthetized animals. In cats, an increase in ventilation was obtained which became considerably smaller, or even vanished completely, when the blood flow to the hind legs was cut off. Section of the lower part of the spinal cord had no influence on the effect of the electrical stimulation on respiration. This was therefore considered to be due to a central effect from substances formed during muscular contractions. In dogs electrical stimulation of anterior roots led in most cases to an increase in ventilation, varying between 8 and 200 percent (on an average 62 percent) and especially was respiration rate influenced, sometimes displaying a great increase, whereas the depth of the respirations was unaffected or slightly increased or decreased. This effect did not disappear after the blood flow to the hind legs had been cut off, but it vanished completely after section of the spinal cord. According to COMROE and SCHMIDT

the increased ventilation with this kind of work in the dog is due to afferent impulses from the legs, thus a different mechanism from that found in cats. It seems doubtful, however, whether the hyperpnea thus obtained in the dog can be considered as a typical work hyperpnea. The extraordinarily strong effect on respiratory rate without a corresponding increase of depth, as seen *e. g.* in COMROE and SCHMIDT's fig. 2, contradicts their interpretation. In the dog, respiration also plays an important rôle in heat regulation, and the heat polypnea is characterized by high respiration rate without increased depth. We have sometimes observed in the dog the sudden appearance of this kind of polypnea without being able to find any direct explanation, and, unless the polypnea subsided in such animals, we have been unable to continue experiments on the regulation of respiration during muscular work. We are therefore unable to accept the results obtained by COMROE and SCHMIDT after stimulation of the anterior roots in the dog, as proof of work hyperpnea. Unfortunately, the gas exchange has not been determined in their experiments, which makes the evaluation still more difficult. This comment also applies to their experiments with passive movements in the dog, where a reflex increase of respiration also was observed. But passive movements were also found to stimulate respiration in cats and in man, and here both rate and depth of the respiration were increased, so that the respiration had the same character as work hyperpnea. But still, the effect in the cat remained after occlusion of the femoral arteries and veins, whereas it disappeared after section of the nerves, or injection of procaine in the knee joints. In man, the passive movements consisted in moving the leg to and fro for about two feet at about 100 times a minute by the aid of a motor. Ventilation increased by 40—50 percent. The effect could be abolished by spinal anesthesia; consequently it must have been of a reflex nature. It cannot be excluded that sensory impulses have to some extent acted like pain, nor that the passive movements reflexly have given rise to increased tension and contractions of different muscles with raised oxygen consumption as a consequence. The increase in ventilation might therefore be due not only to a direct stimulating effect on the respiratory centre, but also to the altered metabolism. The oxygen consumption was unfortunately not determined, but it might be mentioned here that LILJESTRAND and STENSTRÖM (1922), during passive movements of the legs in two subjects, obtained an increase in

the oxygen consumption by 45 and 32 percent respectively, the ventilation at the same time rising by 51 and 104 percent. The last value was undoubtedly in part due to the strong sensory stimulation.

It is pointed out by COMROE and SCHMIDT themselves that the reflexes studied from the working limbs probably play a certain rôle in producing the work hyperpnea, but that it is impossible that they alone can be responsible for it. The effect of the reflexes concerned is probably very moderate, and it will be necessary to consider other possibilities in order to explain work hyperpnea.

Under these circumstances, it seems natural to ask whether the carotid glomus and the aortic glomus might have something to do with the work hyperpnea. It has been definitely established that the carotid glomus, even with an oxygen saturation of the hemoglobin of 95 percent (thus at a value that is normally found not only in anesthetized animals but also in normal man), may react to impulses in the sinus nerve that will stimulate respiration (EULER, LILJESTRAND and ZOTTERMAN 1939). In the case of the aortic glomus, the situation seems to be analogous (GERNANDT 1946). Even a small decrease of the oxygen tension of the arterial blood during muscular work would cause a considerable increase in these impulses and might thereby give rise to a higher activity of the respiratory centre.

Our knowledge of the oxygen tension of the arterial blood is at present very unsatisfactory, owing to technical difficulties, but it seems very probable that the greater oxygen consumption during muscular work will lead to a lowering of the oxygen tension, more pronounced as the amount of work per minute increases. NIELSEN's observation that, during heavy muscular work, inhalation of 45 percent oxygen gives a considerable decrease in ventilation, conforms with this suggestion.

The only investigation about the importance of the sinus region for respiration during muscular work is published by CROMER and IVY (1931), who induced a dog to run, before and after denervation of the sinus. The respiration rate before the denervation was 18.5 during rest, 65.5 immediately after work and, 15 minutes later 59. After denervation the corresponding numbers were 17 before, 59 immediately after and 31, 15 minutes after work. The experiments show that a considerable increase of respiration during work is possible after the sinuses are abolished. The corresponding aortic zones being intact, it is difficult to draw

definite conclusions from these experiments about the rôle of the chemoreceptors during muscular work, especially with regard to the fact that the aortic reflexes in the dog, much more than in the cat, are relatively important in comparison with those of the sinus region.

### Authors' Experiments.

The experiments were mainly performed on cats anesthetized with chloralose (0.05—0.06 g per kg intravenously). In a first series of experiments, the ventilation was recorded from a body plethysmograph (EULER and LILJESTRAND 1936). The animal was placed on its belly and the spinal cord severed at the 12. dorsal vertebra; electrodes of copper wires were introduced around the caudal region of the cord and fixed there. Stimulations were effected by condenser discharges of a strength that was chosen for each animal, and kept constant throughout the experiment. Ventilation was measured after the electrical stimulation had been applied for two minutes. The experiments were repeated under as identical conditions as possible, after denervation of both sinuses. With the aid of a mixture of 7 % oxygen in nitrogen, the complete elimination of the chemical fibres of the sinus regions was obtained: after denervation, only a small and transient increase of ventilation with that gas mixture was permitted (due to stimulation of the aortic bodies). Table 1 gives the results of this series.

Table 1.

*Rest and Work Experiments in Cats before and after Denervation of the Sinus Regions.*

Weight of animal kg	Experimental conditions	Before denervation				After denervation			
		Blood press. mm Hg	Resp. rate per min.	Ventil. l. per min.	Rel. increase in ventil.	Blood press. mm Hg	Resp. rate per min.	Ventil. l. per min.	Rel. increase in ventil.
3.1 ....	Rest Work	98	13.3	0.396		82	12.0	0.269	
		102	14.4	0.682	72 %	76	12.8	0.407	51 %
4.1 ....	Rest Work Rest Work		15.0	0.537			12.4	0.466	
			12.2	1.147	113 %		14.1	0.820	76 %
							14.3	0.561	
							15.7	0.938	67 %
3.0 ....	Rest Work		10.7	0.394			10.6	0.384	
			15.6	1.278	224 %		14.0	0.975	155 %

The table shows that electrical stimulation of the spinal cord has caused in all cases an increase in ventilation which is not very great and corresponds to moderate muscular work. The spinal cord being cut, sensory afferent impulses from the legs cannot play any rôle, and the only possible explanation seems to be that some stimulus has been acting from the circulating blood on the respiratory centre, directly or reflexly. A comparison between the values before and after sinus denervation demonstrates a considerable difference, the increase in ventilation being much smaller after this operation.

At the onset of electrical stimulation of the spinal cord, it was observed that the mean position of the lungs was altered by about 15 ml towards the inspiratory side. Similar observations on cats and dogs have been made by HARRIS (1945) during oxygen-want, starting with 14 % oxygen in the inspired air, as well as by GREEN and SWANSON (1938) in man. In our experiments the effect was still found after sinus denervation.

Experiments were also performed in such a way that electrical stimulation of the hind legs was obtained by an apparatus ("Innervator"), corresponding to the one used by ASMUSSEN, NIELSEN and WIETH-PEDERSEN and constructed for the treatment of paralysed patients. The different electrode was placed on the moistened skin of the back after the hair had been cut, and the indifferent one on the lower part of the abdomen. We used so-called modulated stimulation, consisting of alternating impulses of rhythmically increasing and decreasing strength. The gas exchange of the animals was determined with the aid of small Douglas bags. An abbreviated protocol of an experiment is given below.

Cat, 3.5 kg, was anesthetized by 20 ml 1 % solution of chloralose intravenously. Tracheal cannula and Müller valves. Electrical stimulation with Innervator.

Experimental condit.	Ventilation l. per min. (37°, moist)	Respiration rate per min.	Oxygen con- sumption ml per min., red.	Ventilation ml per ml oxygen consumption
Work .....	0.763	18.0	24.0	31.7
Rest .....	0.458	14.5	16.3	31.2
Work .....	1.020	—	34.9	29.2
Both vagi cut				
Rest .....	0.464	12.0	15.2	30.5
Work .....	0.771	12.2	30.5	22.7
Both sinuses denervated				
Rest .....	0.378	12.0	12.3	30.8
Work .....	0.583	13.5	24.1	24.1

The decrease in the value for ventilation per ml oxygen consumed during work indicates that respiration after vagotomy and sinus denervation is considerably less increased than in the intact animal. The effect was observed even after vagotomy alone, and it therefore seemed desirable to repeat the experiment, but to denervate the sinuses before the vagotomy. The following protocol gives the result.

Cat, 3.7 kg, was anesthetized by 21 ml 1 % chloralose solution intravenously. Arrangements as in preceding experiment.

Experi- mental condit.	Ventilation l. per min. (37°, moist)	Respiration rate per min.	Oxygen con- sumption ml per min., red.	Ventilation ml per ml oxygen consumption
Rest .....	0.409	—	14.8	27.7
Work .....	0.861	17	32.6	28.4
Rest .....	0.394	14.1	13.2	29.8
Work .....	0.705	15.5	30.0	23.5
Both sinuses denervated				
Rest .....	0.305	10.4	12.5	22.5
Work .....	0.609	10.7	29.3	20.8
Rest .....	0.357	10.0	14.3	24.9
Work .....	0.591	10.7	30.3	19.5
Both vagi cut				
Rest .....	0.303	7.7	12.6	22.3
Work .....	0.558	10.4	28.0	17.9
Work .....	0.751	10.0	33.8	22.2

In this case, also, muscular work gives a smaller increase in ventilation after sinus denervation than before. The difference may, to some extent, be explained by the lowering of the respiratory frequency, the dead space being of less importance for the total ventilation at low rates. But this factor can hardly have a deciding influence. Each respiration during rest is about 30 ml. If we reckon  $\frac{1}{3}$  of this value as the volume of the dead space, this is probably too high. From this it can be calculated, however, that the ventilation values after sinus denervation ought to be increased by 50 ml in order to correspond to the values before the operation, the alveolar ventilation in both cases being considered. Ventilation per ml oxygen consumed after denervation would thus become increased to 22.5 and 21.1, *i. e.* they would still be lower than before denervation.

Since the two last mentioned experiments were performed with the spinal cord intact, it was desirable to see whether section of the cord had any effect on the increase in ventilation caused by the electrical stimulation. We found that ventilation rose in the same



way, whether the cord was cut at L1—L2 or not. The result conforms with the observation made by ASMUSSEN, NIELSEN and WIETH-PEDERSEN on their patient with tabes dorsalis; there is this important difference, however, that in the animal experiments all paths in the cord were eliminated. Nor could a diminution of the increase in ventilation during work be observed after bilateral extirpation of the sympathetic chains. The experiments therefore give clear evidence, that the increase in ventilation is not, to any considerable extent, due to impulses travelling from the working muscles through afferent paths. If such impulses are of any importance at all in this connection, it must be very small. Since a great increase in ventilation after muscular work was obtained, even when all influences through the vagi and the sinus nerves were eliminated, it can hardly be doubted that the effect is due mainly to a direct influence on the respiratory centre. The observation that muscular work produces a smaller increase in ventilation after denervation gives some support to the assumption that an "increased excitability" may be brought about by the carotid and aortic bodies. The influence is not great, but it must be remembered that this is not to be expected during moderate work. Stimulation by oxygen-want can then probably not be great, neither is a considerable increase of the impulses in the sinus or depressor nerves, evoked by  $\text{CO}_2$  tension or  $\text{cH}$  probable. As far as experience has shown, we need not reckon with any great reduction of the oxygen tension of the arterial blood, even during heavy work. Nor can the inconstant increase of the carbon dioxide pressure (or  $\text{cH}$ ) be expected to have a decisive influence.

As pointed out above, the experiments in the dog met with special difficulties, periods of hyperpnea sometimes arising without any known stimulation, especially if the cord had been severed. The following experiment in the dog, however, demonstrates analogous conditions to those in the cat, though the irregularities are greater.

Here also, the work hyperpnea after section of the spinal cord is obvious, and it can also be seen, that neither vagotomy nor sinus denervation prevents its appearance. This experiment, like those quoted above, shows that muscular work is accompanied by a considerable increase in ventilation, though the value per ml of oxygen consumed has decreased after denervation of the sinus and vagotomy. This result is also contrary to that of COMROE and SCHMIDT who found that the effect of muscular work on

Dog, 10.7 kg, anesthetized with 100 ml 1 % chloralose solution intravenously. Tracheal cannula and Müller valves. Electrical stimulation of hind legs with "Innervator".

Experi- mental condit.	Ventilation l. per min. (37°, moist)	Respiration rate per min.	Oxygen con- sumption ml per min., red.	Ventilation ml per ml oxygen consumption
Rest .....	3.63	20	57.4	63.3
Rest .....	3.14	34	57.5	65.4
Work .....	5.42	28	105.0	61.6
Shivering, 5 ml 20 % urethane and 2 ml 10 % dial solutions injected intravenously				
Rest .....	3.02	31.5	61.5	58.7
Work .....	7.56	68	186.6	48.4
Spinal cord severed				
Rest .....	3.78	54.5	67.4	67.1
Work .....	14.65	120	266	66.0
Both vagi cut, both sinuses denervated				
Rest .....	3.19	25	70.2	45.5
Work .....	8.41	40	196	43.0

ventilation was abolished in the dog after cordotomy. A priori, it must be considered very unlikely that a primitive reaction like the adaption of respiration to muscular work should be fundamentally different in dogs and cats.

From our experiments, it can therefore be concluded that the increase in ventilation during muscular work can be explained neither by efferent nerve impulses from the working muscles as supposed by ASMUSSEN, CHRISTENSEN and NIELSEN, as well as by COMROE and SCHMIDT, nor by impulses from receptors in the sinus or aortic regions. Insofar as these factors contribute to the increase in respiration during work, these influences must be of moderate or slight importance only. It therefore seems necessary to return to the old hypothesis of a direct chemical stimulation of the respiratory centre, this being taken as the sum of the central nervous mechanisms, whose state of function is correlated to ventilation. We hope to return to these factors in a succeeding communication.

### Summary.

Moderate muscular work, induced by electrical stimulation of the hind-legs in cats or dogs, gives rise to an increase in ventilation, which is not affected by section of the spinal cord or by extirpation of the sympathetic chains. Therefore, the effect on ventilation is

not due to a reflex stimulation from the working muscles, as supposed by some investigators.

Elimination of the sinus and aortic mechanisms did not prevent the increase in ventilation during work, though it became somewhat diminished. It is concluded that the main effect on ventilation during muscular work is due to influences acting through the blood flow on the respiratory centre.

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## The Regulation of the Blood Pressure with Special Reference to Muscular Work.

By

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The greatly increased demand for oxygen during muscular work can be satisfied only if the minute volume of the heart is maintained at a sufficiently high level. The necessary arterial blood pressure, on the other hand, will be primarily dependent on the peripheral resistance, chiefly determined by the conditions of the vascular bed in the working muscles. In order that the general functions of the organism remain unimpaired, it must neither fall to such levels as may invalidate the blood supply to the central nervous system, nor rise to levels which would incur a heavy load on the heart. For the maintenance of an adequate blood pressure level, some sort of regulatory influence must of necessity come into play. As a matter of fact, only fairly moderate alterations of the blood pressure level have been found even during heavy work. Usually a moderate increase is met with, though sometimes an insignificant lowering has been observed. Thus KAUFMANN (1892), recording the pressure in the carotid artery of the horse, usually found a small decrease when the animal was walking, and ZUNTZ and HAGEMANN (1898) recorded a corresponding effect: on an average, the pressure fell from 155 to 143 mm. In the dog, TANGL and ZUNTZ (1898) found an average rise of 6 mm during light work, and 23 mm during heavy work. At the onset of work there was often a slight decrease in pressure lasting a few seconds.

With regard to man, several authors have observed an increase of the systolic pressure during muscular work (Cp. TIGERSTEDT

1922), but these results give no definite information as to the variations of the mean pressure. LILJESTRAND and STENSTRÖM determined the systolic as well as the diastolic pressure within ten seconds after the termination of a period of walking and running (1920), and swimming (1919), and found a much greater increase of the systolic than of the diastolic pressure, so that only a moderate rise of the mean pressure resulted. On the other hand the systolic pressure fell rapidly after cessation of the work (LILJESTRAND 1932); the mean values during the work itself must have been higher than those observed. During rest and work on the Krogh bicycle ergometer, LILJESTRAND and ZANDER (1928) found, in two healthy subjects that, though the systolic pressure rose considerably in proportion to the amount of work performed the diastolic values increased only slightly or varied irregularly. In one of the subjects, the mean pressure rose from a resting value of 93 mm, corresponding to an oxygen consumption of 0.25 l. per minute, to a maximum of 150 mm (at an oxygen consumption of 2.5 l. per minute); in the other, the corresponding values were 89.5 mm at rest (0.21 l. oxygen consumption per minute) and 125 mm (1.6 l. oxygen per minute). The rise in the mean pressure is thus very moderate. An increase of the metabolism by 100 percent is accompanied by an increase of the blood pressure by 6—7 percent. Similar results have later been obtained by BOCK, VANCAULERT, DILL, FÖLLING and HURXTHAL (1928) and by ELDAHL (1933). In both these investigations the work was performed on a bicycle ergometer.

It seems very probable, however, that the effect on the blood pressure varies somewhat with the kind of work performed. This is indicated by an investigation of COTTON, LEWIS and RAPPOPORT (1917). The work consisted in swinging 10 kg dumb-bells from the floor to the full stretch of the arms above the head and down, in two, three, four or more seconds and repeating this procedure a varying number of times. The work had thus a pronounced statical component. This may explain why the systolic and mean pressures rose after cessation of the work and attained a maximum about a minute after the work. After working on a bicycle, on the other hand, BOCK, VANCAULERT, DILL, FÖLLING and HURXTHAL observed a rapid fall of the systolic and mean pressures, and LILJESTRAND as well as ELDAHL, had the same experience.

When the oxygen consumption rises, the blood flow or the minute volume of the heart is proportionately increased. During

work on the bicycle ergometer a rise in metabolism by 100 percent is usually accompanied by an increase in the blood flow of about 50 percent. If the state of contraction of the blood vessels were to remain constant, the mean blood pressure must rise by a similar percentage. As has been pointed out above, the increase observed is only about  $1/8$  as great. This example gives an idea of the considerable adjustments in the width of the vessels that actually take place during muscular work.

### Authors' Investigations.

In these investigations the arterial blood pressure in the carotid artery of various animals has been recorded with a mercury manometer. This vessel was chosen in order to retain the blood supply to the hindlegs intact while they were performing the muscular work. It might be argued that, by tying off one carotid artery, conditions must be greatly altered with regard to blood pressure and blood flow in the sinus region of that side, and that thereby interactions on the circulation as a whole, or on the power of regulation, might be expected. This source of error has been considered but comparative observations with recording made from another vessel, *e. g.* the femoral artery, have not substantiated the fear, that the onesided tying off a carotid artery would have influenced our results to any appreciable degree. The reason lies undoubtedly in the compensational activity exercised by the remaining similar reflexogenic zones. Since the main object of this study is to elucidate the interaction of muscular work on circulation, as far as the blood pressure is concerned, it has been considered of primary interest to investigate the effects under different experimental conditions of such alterations in the composition of the blood gases as might be expected during muscular work. Both the carbon dioxide and the oxygen pressures might be affected and give rise to local as well as reflex and central effects. We have therefore tried the effects of inhalation of gas mixtures with varying carbon dioxide and oxygen contents. The following methods have been used in this connection:

- 1) Denervation of the baro- and chemoreceptor zones which might reflexly influence circulation.

- 2) Specific elimination of certain parts of this reflex mechanism by means of small amounts of ergotamine (0.15—0.2 mg per kg).

- 3) Elimination of the central mechanisms in the brain and the medulla (decapitation) with or without elimination of the spinal centres (pithing).

Denervation of the baroreceptor and chemoreceptor zones has been performed by tying off the tissue between the internal and external carotid arteries and by vagotomy, the depressor nerves in cats and dogs passing more or less completely in the vagosympathetic trunk. In rabbits the depressor nerves were cut in addition.

The experimental variations of the gaseous tensions in the blood and the tissues were obtained by letting the animals inspire suitable

gas mixtures. In order to obtain gradual accumulation of carbon dioxide the animals were made to breathe in a closed spirometer system, which was filled with oxygen; if oxygen-want was to be established, the system contained air from which the carbon dioxide was absorbed in the usual way by soda lime. Carbon dioxide accumulation and oxygen-want could easily be produced simultaneously, if the system contained air but no absorption of carbon dioxide took place. For rapid alterations gas mixtures were given from bags, which were either connected to the inspiratory side of the Lovén valves or — in case of artificial respiration— with the Starling pump. A state of hypoeapnia was sometimes produced by artificial overventilation with gas mixtures poor in carbon dioxide.

To control the effects of the gas mixtures, alveolar air samples were taken frequently at the end of expirations (cp. EULER and LILJESTRAND 1936), and analysed.

Most of the results given below have been obtained in cats under chloralose anesthesia (0.05—0.06 g per kg intravenously). Sometimes corresponding experiments have been performed in dogs and rabbits. When the results from these animals have been at variance with those from the cat, it has been mentioned in the next.

### 1. The Effect of varying Carbon Dioxide Tensions on the Blood Pressure.

In animals with intact blood pressure reflexes from the sinus and aortic regions, inhalation of air or oxygen with a moderate percentage of carbon dioxide (below 10 %) usually produced an insignificant or no effect on the blood pressure. If the carbon dioxide percentage in the inspired air increased continually and the oxygen percentage was maintained at a high level, we observed in some cases a characteristic sequence of effects on the blood pressure. Usually there was an initial moderate lowering of the pressure, followed later by an increase, which as a rule continued until there were very high values of carbon dioxide in the inspired and the alveolar air. The initial lowering of the pressure is probably due to a peripheral local dilatation which is only incompletely compensated. The secondary rise must be the result of a stimulating effect on the vasomotor centre, acting partly directly, and partly via the chemoreceptors. In the experiment given in fig. 1, where the alterations were very marked, the pressure started at 127 mm with the alveolar carbon dioxide at about 5 %. At first a decrease of the pressure to 104 mm occurred with the alveolar carbon dioxide at about 8 %, but when the alveolar carbon dioxide had risen to 15—20 %, the initial level of the pressure had again been reached, and then it rose continuously, so that it

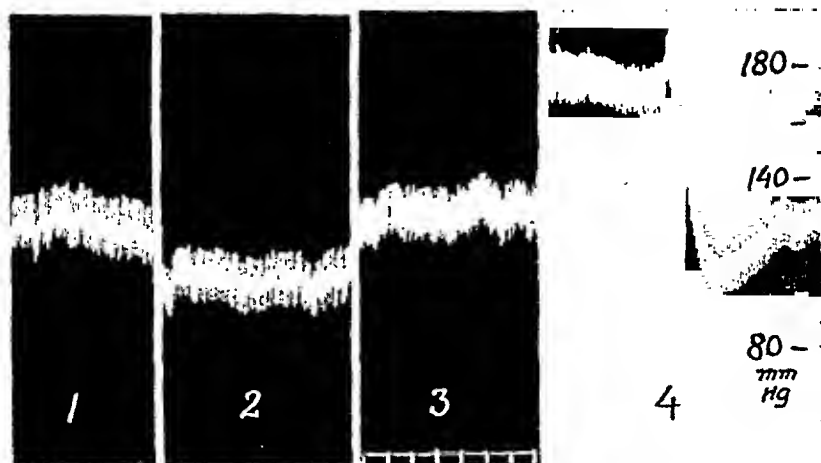


Fig. 1. Cat, chloralose, blood pressure. Animal breathing oxygen in close system with accumulation of carbon dioxide.

1. (10.47) 5 p.c. alveolar  $\text{CO}_2$
  2. (11.00) 8 " " "
  3. (11.54) 20 " " "
  4. (12.50) 38 " " " Shift to air breathing.
- Time 30 sec.

was 177 mm when the alveolar carbon dioxide had attained the value of 38 %.

In other cases, no definite effects on the blood pressure were obtained in spite of the fact that the alveolar carbon dioxide had risen to 25 %, which must signify a very efficient regulation. If this interpretation is correct, a definite effect of carbon dioxide is to be expected, if the regulating reflex mechanisms are eliminated. This has always been our experience. In one experiment, where the variations of the blood pressure under carbon dioxide accumulation were only about 10 mm with the reflex regulation intact, after denervation carbon dioxide caused a very marked effect. The pressure started at 170 mm at about 6 % alveolar carbon dioxide; it sank to 148 at 8 % carbon dioxide and then rose continually to 232 mm at about 28 % carbon dioxide in the alveolar air.

Whether the reflex mechanisms were maintained or eliminated, the change from air rich in carbon dioxide to atmospheric air was followed by a quick return of the blood pressure, which could even for some time sink below the initial level as illustrated in fig. 1. A drop was observed also, in such cases where the blood



pressure remained constant during the accumulation of carbon dioxide, and in this event it was reversible.

As shown by ROTHLIN (1923), HEYMANS and REGNIERS (1929), and WRIGHT (1930), small doses of ergotamine cause an elimination of reflexes from the baroreceptors. The mechanism of this action has been interpreted in different ways. Thus HEYMANS and REGNIERS have advanced the opinion that the elimination of the reflex is due to a blocking of the peripheral vasomotor action, which is known to occur with high doses of ergotamine or ergotoxine, whereas WRIGHT has produced evidence to show that the effect is on some central mechanism on the afferent side of the vasomotor centre. The latter view seems to gain support by the findings of EULER and SCHMITERLÖW (1944). These authors observed that the reflex vasomotor actions elicited by stimulation of the chemoreceptors of the sinus region by means of oxygen-want were not suppressed by small doses of ergotamine, though the reflexes evoked by pressure changes in the sinus were abolished.

Small doses of ergotamine (or ergotoxine) act not only on the reflexes elicited from the baroreceptors, but also cause a profound change in the vasomotor response to carbon dioxide. As shown by GANTER (1926), KOHN (1932) and HEYMANS and BOUCKAERT (1933) the normal rise in blood pressure called forth by asphyxia or by administration of gas mixtures rich in carbon dioxide, is replaced by a marked lowering of the pressure after moderate or even small doses of ergotamine. This effect has also been noticed recently by GERNANDT and ZOTTERMANN (1946). Since the small doses of ergotamine do not suppress the ability of the blood vessels to react to vasoconstrictor impulses, as mentioned above it seems probable that the chief action of ergotamine is that of blocking the vasomotor centre to the action of carbon dioxide, which then only exerts its usual local vasodilating action, revealing itself in a fall of blood pressure.

When a dose of ergotamine, sufficient to cause a selective elimination of the baroreceptor reflexes was given, as in the experiments of EULER and SCHMITERLÖW, a reversal of the action of carbon dioxide on the blood pressure was noted, in conformity with the observations of previous authors. The inhalation of a continuously increasing percentage of carbon dioxide in the inhaled gas mixture now produced a much greater primary lowering of the blood pressure than the one observed after complete de-



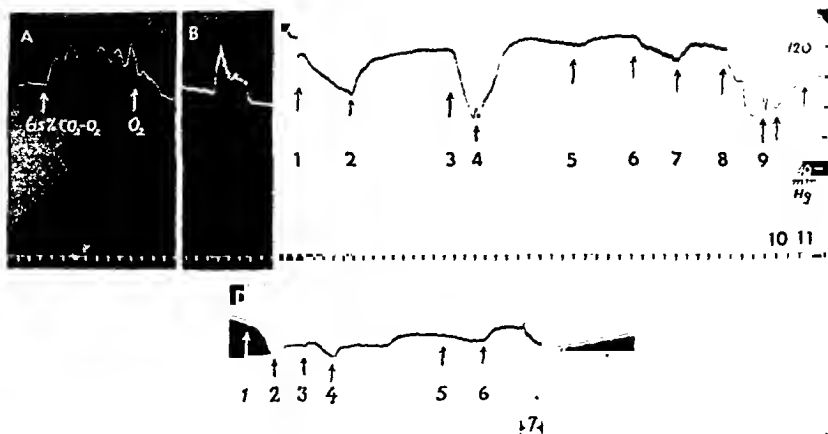


Fig. 3. Spinal cat, blood pressure, artificial respiration.

A. Shift from  $O_2$  to 6.5 p.c.  $CO_2$  in  $O_2$ .

B. Muscular work, air.

C. 1. Artificial respiration decreased.

2. Art. resp. increased.

3. 6.8 p.c.  $O_2$  in  $N_2$ .

4. Convulsions, shift to air.

5.  $O_2$ .

6. 6.5 p.c.  $CO_2$  in  $O_2$ .

7. Air.

8. Art. resp. increased.

9. Convulsions.

10. Continued hyperventilation 6.5 p.c.  $CO_2$  in  $O_2$ .

11. Art. resp. decreased, air.

D. (After Pithing) 1. and 3. 6. 8. p.c.  $O_2$  in  $N_2$ . 2., 4., 6., Air. 5. 6.5 p.c.  $CO_2$  in  $O_2$ .

7. Muscular work.

Time 30 sec.

destroying the brain and the spinal cord. Also, in this case, a lowering of the blood pressure is constantly found after carbon dioxide. This is sometimes also the case after decapitation alone; in other cases, however, a stimulating effect of carbon dioxide can be observed, probably via "the spinal vasomotor centres". Opposing effects of carbon dioxide have also been observed. At a relatively high pressure a moderate drop may occur; but hyperventilation with the same carbon dioxide mixture can give an increase of the pressure, after it had previously been lowered by hyperventilation with air (Fig. 3).

A diminution of the carbon dioxide tension by overventilation with air or oxygen has quite regularly given a moderate fall of the blood pressure. This illustrates the wellknown fact that the normal carbon dioxide tension is an important contributory factor for the maintenance of the normal pressure.

## 2. The Effect of Variations in the Oxygen Tension on the Blood Pressure.

In contrast with carbon dioxide, which exercises a stimulating influence on the blood pressure partly directly, and partly through a reflex effect from the chemoreceptors of the sinus and aortic regions, and thus is able, more or less, to compensate for the peripheral vasodilating effect, oxygen-want causes a rise in the blood pressure which is almost entirely due to a reflex effect via the stimulated chemoreceptors. The central effect is usually of the opposite nature and leads to a fall of the blood pressure; in the cat, this is the typical reaction to oxygen-want, when the reflexogenic zones have become eliminated. Their high sensitivity for oxygen-want makes it easy to understand why even a low desaturation of the blood (or a decrease of the blood supply through the glomerus regions), may bring into play this reflex. During usual anaesthesia, as a rule, a similar moderate decrease of the oxygen saturation of the blood is met with, and in consequence oxygen inhalation causes an elimination of the reflex increase of the blood pressure, as well as of the pulse rate (EULER and LILJESTRAND 1942). On the other hand, oxygen-want, as has been mentioned, after elimination of the reflex mechanisms, causes a lowering of the blood pressure, whereas oxygen inhalation now leads to an increase (Fig. 4).

The effects just mentioned relate to conditions found in the cat, but in principle the same results were obtained in dogs and rabbits. In the dog, in most cases a certain stimulating action of oxygen-want on the centres was met with, which was demonstrated after elimination of the reflexogenic zones.

Under normal conditions, the effect of oxygen-want is so effectively compensated by the blood pressure regulation that inhalation of gas mixtures poor in oxygen, *e. g.* 10 % oxygen in nitrogen, does not cause any marked increase of the blood pressure. After selective elimination of the baroreceptor reflexes with the chemoreceptor reflexes partly maintained, the effect of oxygen-want on the blood pressure can be made manifest. In the dog, this can be obtained through mechanical denervation of the baroreceptors alone, an operation which is difficult to perform in the cat. As mentioned before, we have the means of eliminating — by the aid of ergotamine — the baroreceptor reflexes and the direct or indirect

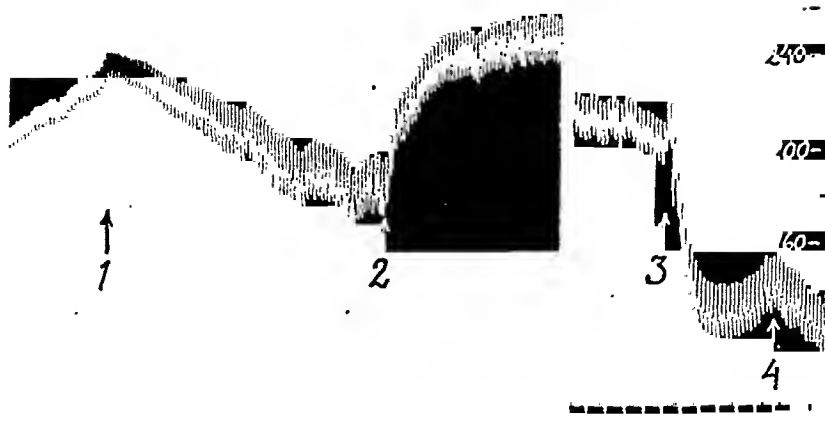


Fig. 4. Cat, chloralose, blood pressure. Both sinus regions denervated, both vagi cut in the neck.

1. Shift from  $O_2$  to air.
2. Shift from air to  $O_2$  with accumulation of carbon dioxide.
3. Shift from  $O_2$  with 14.2 p.c.  $CO_2$  to  $O_2$  (40 min. later).
4. Air.

Time 30 sec.

effects of carbon dioxide on the vasomotor centre. Thus the animal, after having been treated with an adequate dose of ergotamine (0.1—0.2 mg/kg) reacts to oxygen-want with a marked increase of the blood pressure, demonstrating that the chemical reflex excitability for oxygen-want is maintained though the baroreceptor reflex regulation is lost (fig. 2). If oxygen-want is combined with carbon dioxide accumulation, an antagonistic factor comes into play, since, after elimination of the baroreceptor regulation with ergotamine, the effect of carbon dioxide is a general lowering of the blood pressure. Consequently, an asphyxial stimulation may lead to a fall in the blood pressure after a small dose of ergotamine in spite of the reflex mechanism for oxygen-want being intact.

After decapitation and destruction of the spinal cord, respectively, the effect of oxygen-want on the blood pressure is to cause a decrease; in the cat, as mentioned before, this is the rule after elimination of the reflexogenic zones.

A comparison between the effects of different gas mixtures on the blood pressure under different conditions will be found on p. 295.



Fig. 5. Rabbit, urethane, blood pressure. A. muscular work. B. Same after denervation of both sinus regions and cutting the depressor nerves. C. Same after section of the spinal cord at L1—L2. Time 10 sec.

### 3. The Effect of Muscular Work on the Blood Pressure.

Muscular work was obtained by means of electrical stimulation with the "Innervator" as described in an earlier paper (EULER and LILJESTRAND 1946). Both modulated and unmodulated (ordinary faradic) stimulation was tried.

#### a) Normal animals.

With the onset of the work in most cases a moderate initial lowering of the blood pressure was observed, soon, however, followed by a more or less rapid return to the original level, at which the pressure remained nearly constant, though a moderate rise above that level was often found as the work continued (fig. 5—7). The primary lowering was most marked in the rabbit but less conspicuous, or of shorter duration, in the cat and dog, indicating that the counter-regulation was more efficient in those animals. During unmodulated stimulation, which caused a sustained contraction of the muscles, a more or less considerable rise of the blood pressure was observed, probably mainly due to mechanical factors. When the modulated stimulation was used, the pressure remained on the whole unaltered. In those cases, however, where a moderate rise or a decrease of the pressure had taken place during stimulation, it soon returned to the original value obtained before stimulation.

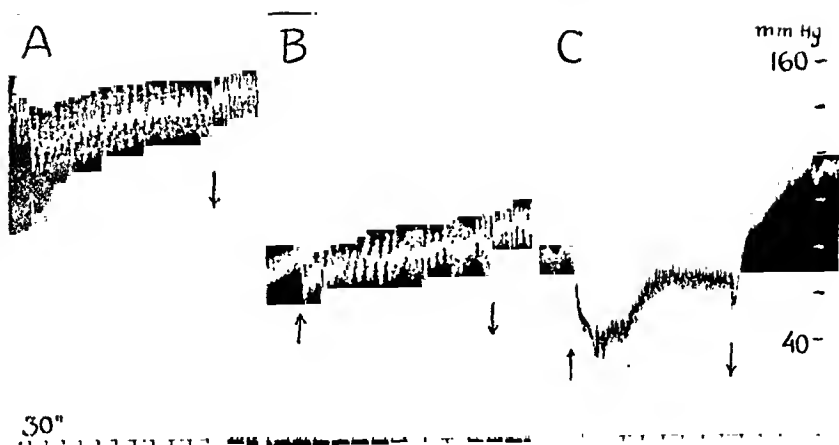


Fig. 6. Dog, chloralose, blood pressure. A. Muscular work. B. Same after section of the spinal cord at L1—L2. C. Same after denervation of both sinus regions and vagotomy in the neck. Time 30 sec.

*b) The Effect of Muscular Work on the Blood Pressure after Elimination of the Sinus and Aorta Mechanisms.*

As illustrated in fig. 5—7, muscular work, after elimination of the reflexogenic zones, provoked a more or less pronounced lowering of the blood pressure according to the degree of muscular activity. This was observed in cats as well as in dogs and rabbits. The decrease in the pressure was seen immediately after the work had started, but in contrast to the animals with intact regulation, the fall of pressure was very incompletely compensated and remained for a considerable time at the low level throughout the period of muscular work. In several cases a slow increase of the blood pressure was observed as work went on and this tendency was especially obvious in experiments where a rise above the original value was observed after the work had stopped, a phenomenon that will be referred to later.

The considerable lowering of the blood pressure during work in animals, the reflex regulatory mechanisms of which had been eliminated, is undoubtedly due to the wellknown dilatation of vessels in the active muscles that takes place during work. At the same time, the result demonstrates that the vasodilatation under normal conditions is so effectively compensated by the functioning of the regulating mechanisms that only a very in-

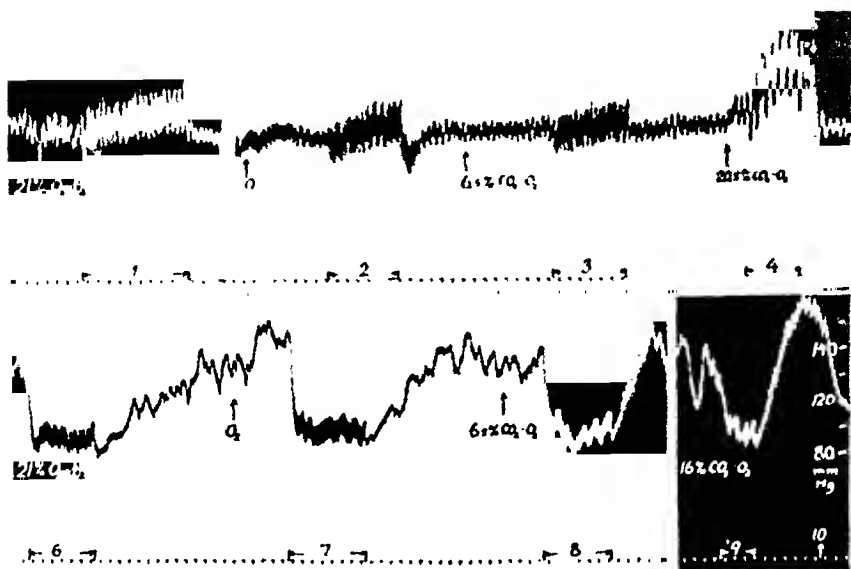


Fig. 7. Cat, chloralose, blood pressure, 1.—4. Muscular work during breathing of 1) air 2) oxygen 3) 6.5 p.c.  $\text{CO}_2$  in  $\text{O}_2$  and 4) 20.5 p.c.  $\text{CO}_2$  in  $\text{O}_2$ . 5. Shift to air. 6.—9. Muscular work during breathing of 6) air, 7)  $\text{O}_2$ , 8) 6.5 p.c.  $\text{CO}_2$  in  $\text{O}_2$ , 9) 16 p.c.  $\text{CO}_2$  in  $\text{O}_2$  after denervation of both sinus regions and vagotomy. 10. Shift to air. Time 30 sec.

significant alteration of the blood pressure ensues. From our knowledge of the nature of this compensatory mechanism, it can be concluded that muscular work must be accompanied by a reflex increase in the activity of the vasomotor and cardio-accelerator centres, and probably also with an increased secretion from the adrenal medulla; the vagal tone on the other hand must decrease. This conforms with earlier results by HERING (1895), AULO (1911) a. o. who attributed the rise in pulse rate during muscular work chiefly to an increased tone of the accelerator nerves and a lessened tone of the vagi. CANNON (1929), MEYTHALER and WOSSIDLO (1937) and RAAB (1943) a. o. have given further evidence in favour of the view that muscular work is accompanied by an increased secretion of adrenaline and symphatin respectively.

As already mentioned a rise of the blood pressure, sometimes very considerable, has been observed in numerous cases after cessation of work. Such a secondary rise has been found in the cat, as well as in dogs and rabbits (fig. 5—7). Since this effect did not occur in animals with intact regulation of the blood pressure, it



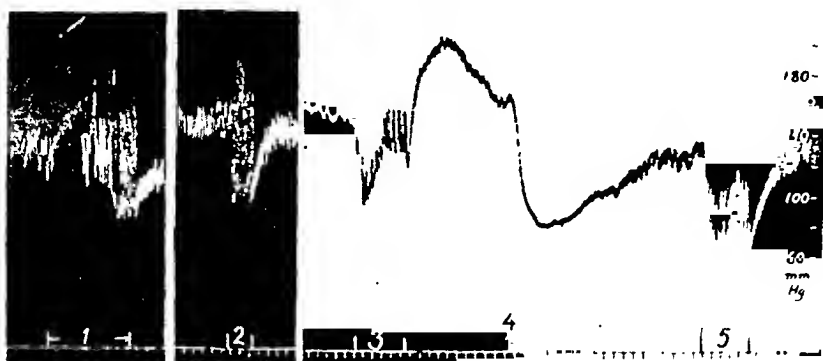


Fig. 8. Cat, chloralose, blood pressure. 1. Muscular work, air, 2. Same in  $O_2$  with accumulation of  $CO_2$  (alveolar  $CO_2$  10.8 p.c.). 3. after denervation of both sinus regions and vagotomy, muscular work (alveolar  $CO_2$  14.6 p.c., alveolar  $O_2$  10.1 p.c.). 4. Oxygen. 5. Same work as at 3, but breathing oxygen. Time 30 sec.

seems probable that under normal conditions it is compensated and therefore does not appear. It seems reasonable to assume that this increase is due to a central effect of carbon dioxide which appears to be the only factor (apart from increased oxygen pressure during oxygen-want) that has been found to give a rise in the blood pressure in animals, deprived of their reflexogenic zones. Fig 7:9 shows that the secondary rise appears also with a gas mixtures rich in oxygen, if the carbon dioxide percentage is sufficiently high. It may even become very considerable in spite of a certain oxygen-want as is illustrated in fig. 8:3. (Cp. also under d.) As illustrated by fig. 6, the effect also is observed after section of the spinal cord at L1—L2, that is, above the area of stimulation. This operation has in no way led to other obvious alterations of the behaviour of the blood pressure during muscular work; consequently, nervous impulses originating from the working muscles themselves and passing to the central nervous system are of little importance for the blood pressure regulation. This is in contrast to the conclusions of ASMUSSEN, NIELSEN and WIETH-PEDERSEN (1943). As has been shown in another paper (EULER and LILJESTRAND 1946), the same holds true for the regulation of respiration during muscular work.

The increase in blood pressure, observed during muscular work produced by unmodulated stimulation, was enhanced by denervation of the sinus and aortic regions, as was to be expected. The increase persisted as long as the work was in progress and was

followed by a fall to the original level, or temporarily even below it.

*c) Effect of Muscular Work on the Blood Pressure after Decapitation and Destruction of the Central Nervous System.*

In decapitated animals, with the spinal cord severed at the 2nd cervical vertebra, muscular work was accompanied by a moderate increase of the blood pressure. If the spinal cord was destroyed, a fall of the blood pressure during work was observed which lasted for several minutes after the work had ceased.

*d) Effect of Muscular Work on the Blood Pressure during Inhalation of Gas Mixtures with varying Oxygen and Carbon Dioxide Percentages.*

In animals with intact reflex regulation of the blood pressure no obvious effects on the blood pressure were obtained by muscular work if, instead of air, pure oxygen or a mixture of 6.5 % carbon dioxide in oxygen were inhaled (fig. 7). During inhalation of gas mixtures of 15—20 % carbon dioxide in oxygen, or mixtures rich in carbon dioxide and poor in oxygen, which alone resulted in an increase of the blood pressure, this effect was probably enhanced by muscular work (fig. 7: 4).

The rise of blood pressure after work, mentioned under b), in animals with the reflexogenic zones eliminated, could not be elicited in all cases, but it could usually be made manifest in certain ways. Thus fig. 7 and 8 show obvious differences with regard to the blood pressure curve in connection with the inhalation of gas mixtures of varying composition. Whereas inhalation of air or oxygen gave on the whole similar results, 6.5 % carbon dioxide in oxygen, and still more 16 %  $\text{CO}_2$  in  $\text{O}_2$ , caused a much quicker return of the pressure to the original level; in the last case it even exceeded this. It seems probable that the secondary rise in the blood pressure after the cessation of work in animals with the sinus and aortic regions denervated has been due to an effect of accumulation of carbon dioxide or rather acid metabolites secondary to oxygen lack within the reacting cells of the centres. If this assumption is correct it is to be expected that the rise should vanish after treatment with ergotamine, which in fact was the case, as seen in fig. 2 and 9. Not only did the blood pressure fail to exceed the resting level after the cessation of work in ergotamine animals, but the return to normal was rather

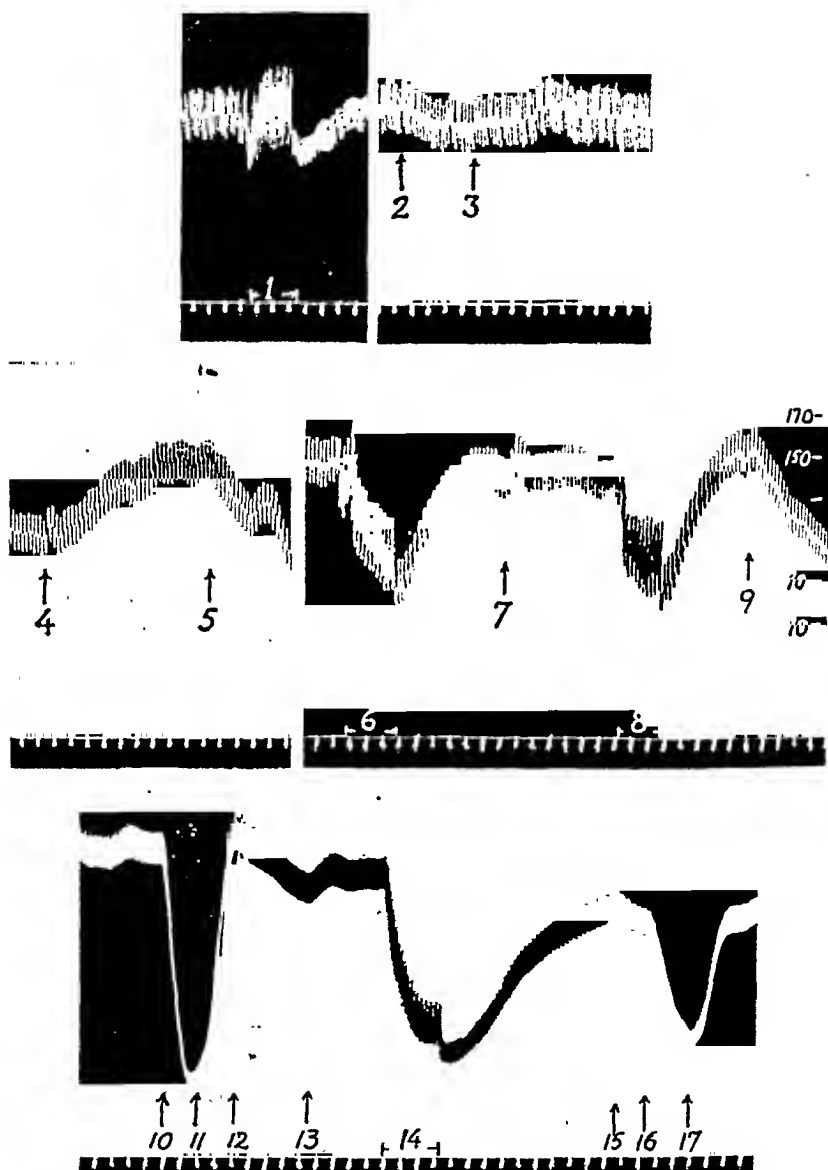


Fig. 9. Cat, chloralose, blood pressure. 1. Muscular work. 2. From air to  $O_2$ . 3. 16 p. c.  $CO_2$  in  $O_2$ . 4. (after denervation of both sinus regions and vagotomy) shift from  $O_2$  to 16 p.c.  $CO_2$  in  $O_2$ . 5.  $O_2$ . 6. Muscular work,  $O_2$ . 7. 16 p.c.  $CO_2$  in  $O_2$ . 8. Muscular work. 9.  $O_2$ . 10. (after 0.2 mg Gynergen/kg), 16. p.c.  $CO_2$  in  $O_2$ . 11. and 13.  $O_2$ . 12. and 15. air. 14. Muscular work. 16. 6.5 p.c.  $CO_2$  in  $O_2$ . 17. air. Time 30 sec:

slower. In addition the decrease in blood pressure during work was very accentuated after ergotamine. These results therefore support the interpretation suggested above as to the cause of the rise in blood pressure during and after work in the denervated animals.

In order to obtain an inhibition of the pressure-increasing effect of carbon dioxide, as well as a lowering for some time after the cessation of work, a dose of ergotamine of about 0.15—0.2 mg per kg is needed. A dose of 0.1 mg per kg, as a rule, abolishes the blood pressure rise during clamping of the carotids but the rise of the pressure during carbon dioxide accumulation has not been entirely absent in all cases.

*Summary of the Effects on the Blood Pressure of Muscular Work and of Inhalation of Gas Mixtures of varying Composition.*

(+ = rise, — = lowering of the pressure. If the signs are in brackets, the effect is small. +— or —+ signifies consecutive effects, ± denotes inconstant results.)

Normal	After denervation of the sinus and aortic mechanisms	After ergotaminization 0.15—0.2 mg per kg.	After decapitation	After decapitation and destruction of the C.N.S.
Gas mixtures rich in CO <sub>2</sub> (—) (+)	— +	—	+	—
Overventilation with air or oxygen .....	—	—	—	—
Oxygen inhalation .... (—)	(+)	—	(+)	(+)
Oxygen want .....	(+)	+ + <sup>1</sup>	—	—
Muscular work .....	(—) (+)	—	±	—

<sup>1</sup> If the hypoxemia is considerable, the blood pressure decreases.

### Discussion.

The effect of muscular work on the blood pressure is the final result of several factors in opposite directions and with varying strength. A number of such factors have been studied in this paper. In accordance with earlier observations, it has been found that accumulation of carbon dioxide, like other acids (Cp. ATZLER and LEHMANN 1927), within certain limits leads to a peripheral dilatation of the vascular bed. Such an accumulation, which will occur in consequence of the increased metabolism, also acts as a stimulus to the vasomotor centre both directly and reflexly via the sinus and aortic mechanisms. A decrease of the oxygen tension also causes peripheral dilatation (cp. KROGH 1922), and

this effect may be counteracted by a reflex stimulation of the vasomotor centre, elicited through the chemoreceptors. The direct effect on the centre, however, is mainly depressant. Mechanical factors, *e. g.* the squeezing out of blood from the muscles in certain kinds of work, may also be of importance. The most prominent rôle in the regulation of the blood pressure is certainly played by the baroreceptors. Usually their influence is so great that the pressure is only altered to a small extent. Sometimes, however, the other factors mentioned may have so strong an influence that the pressoregulation through the baroreceptors is insufficient to prevent a considerable rise, or in other cases a moderate fall.

Though much study is needed in order to explain in detail the effects of muscular work on the blood pressure, our results seem to indicate certain possibilities. The first reaction to muscular work, noted by other authors as well as by ourselves, is a primary lowering of the pressure for a short while. It seems probable that this is due to the local effect of carbon dioxide accumulation and oxygen-want. It takes some time, however, before the effect is carried by the blood to the chemoreceptors and the centre, especially if the blood flow is not free as in some cases of statical work. There may also be a certain period of latency in the reflex apparatus itself (Cp. the relatively slow effect when the carotids are clamped and the drop below the original level in fig. 1: 4). The response in the form of a general increase in the tone of the vasomotor centre will therefore often be too slow to prevent a marked fall at first. Soon, however, the pressure rises again, though sometimes it remains somewhat lower than during rest. This would seem to be the natural outcome, if the baroreceptors alone were responsible for the regulation of the tone of the vasomotor centre. It may be added in this connection that ASMUSSEN, NIELSEN and WIETH-PEDERSEN (1943), contrary to the assumption of pressoreflexes being a link in the regulation of the arterial blood pressure during muscular work, make the objection that "it is not easy to understand how the reflex reactions can be brought about at the onset of the work, as normally no initial fall in the arterial blood pressure is observed". From the experiments described in this paper it is obvious that, as a rule, an initial fall does occur, and the results obtained after denervation of the baroreceptive zones seem to leave no doubt as to the great importance of the baroreceptors in maintaining the pressure during

muscular work. The great rôle played by the pressure regulating system during muscular work is also borne out by the fact that after thoraco-lumbar sympathectomy in man muscular work is accompanied by a drop in diastolic blood pressure of as much as 30 mm Hg (LORD and HINTON, 1945).

Often, the blood pressure during work rises continually for several minutes and even exceeds the resting value. The explanation seems to be that this is the outcome of the chemical influences acting directly or reflexly on the centre. Fig. 5 and 6 demonstrate that such a gradual increase is sometimes observed during work after denervation of the sinus and aortic regions. When the work was ended and the peripheral effects of the metabolites on the local tone of the vessels rapidly decreased, a considerable rise in the blood pressure above the resting value was found in the experiments on the dog and the rabbit. A similar but lesser effect is also observed in the cat (Fig. 7). It is to be remembered that, in the cat, oxygen deficiency appears to have a stronger depressant effect on the vasomotor centre than in the dog, while the chemoreceptors may possibly play a greater rôle in the cat, which might explain the differences in results found between the species of animals investigated. It is difficult in these cases to find an explanation for the rise of the pressure above the resting value other than through the mediation of the blood.

It has been pointed out before (p. 282) that relatively low tensions of carbon dioxide in the intact, as well as in the denervated animal, cause a decrease of the blood pressure whereas higher concentrations cause an increase. In the former case, the peripheral effect dominates; in the latter, the central. It is therefore to be expected that the central influence will become relatively greater as the amount of the work increases. This would seem to conform with the observations quoted in the beginning of our paper: that the blood pressure during light work is often maintained at a low level — sometimes even lower than during rest — but that it is raised during heavy work, and also that the rise is somewhat in proportion to the amount of work done per minute. It is obvious that oxygen want in the intact animal acts essentially in the same direction as accumulation of carbon dioxide, the depressant direct effect on the centre being under ordinary conditions, overcompensated by the reflex action through the chemoreceptors. It is also conceivable that accumulation of acid metabolites, secondary to oxygen lack, may have a stimulating influence on the

blood pressure regulating centres, and in this case the effect should appear in the sinus-denervated animal as well.

It might be argued that carbon dioxide provoked a blood pressure rise similar to that found in denervated animals after the cessation of muscular work, but only in rather high concentrations (about 15—20 % in the inspired air), and that such concentrations are unlikely to occur in the blood during or after work. But it is quite possible that considerable alterations in the reaction may take place within the cells of the vasomotor centre during work, even if the corresponding changes in the blood are relatively small. A similar situation with regard to the respiratory centre has sometimes been assumed.

If the blood flow through the working muscles is impaired, it is to be expected that the central effects of carbon dioxide accumulation and oxygen-want will appear at the moment of establishment of better conditions for the circulation. This seems to explain in part — together with the disappearance of the peripheral dilatation — the great increase in the blood pressure observed on cessation of work in the denervated animals having a low and rather insufficient blood pressure during work. It might also explain the high pressure observed after statical work (LEWIS and collaborators). There seems to be little doubt according to the investigations of LINDHARD (1920) that during statical work the circulation may easily be hampered to a greater or lesser degree.

### Summary.

The effect of various gas mixtures and of muscular work has been studied on the blood pressure of cats and dogs in chloralose anaesthesia and in rabbits under urethane.

A gradual increase of the carbon dioxide content of inspired oxygen caused relatively slight changes in the blood pressure of the cat. After elimination of the sinus and aortic mechanisms a primary fall and a secondary rise of considerable magnitude ensued, the former due to peripheral vasodilatation and the latter due to central stimulation. Similar though less pronounced changes were sometimes observed also before the denervation.

The rise in blood pressure by chemoreflex action of oxygen want was increased after elimination of the baroreceptor mechanism by means of ergotamine. Denervation of the reflexogenic areas reversed the effect of oxygen lack in the cat.

Moderate muscular work, induced by electrical stimulation of the hind legs, caused little change in the blood pressure apart from an initial transitory fall. The same result was obtained after section of the spinal cord. In animals deprived of their sinus and aortic reflexes the blood pressure was greatly lowered during muscular work. A rise above the resting level was sometimes observed after the termination of work. This rise was accentuated or could be provoked by inhalation of gas mixtures rich in carbon dioxide and poor in oxygen. It was often considerable, when the sinus and depressor mechanisms had been eliminated.

It is concluded that the blood pressure during muscular work is maintained at an adequate level chiefly by the mediation of the baroreceptor reflex mechanism. In addition the blood pressure may be influenced by central actions secondary to carbon dioxide accumulation or deficient oxygen supply or both.

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## Observations on the Pulmonary Arterial Blood Pressure in the Cat.

By

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The well-known regulatory influence exerted on the general blood pressure by impulses deriving from the sinus and aortic reflexogenic zones raises the question as to whether the pulmonary arterial blood pressure is subject to regulatory forces of a similar or different kind. The lungs, being placed in series with the general circulation, would scarcely be dependent on the maintenance of a certain blood pressure level in order to ensure the blood supply to the organ itself, as is the case for a number of organs in the general circulation, notably the central nervous system and the kidneys. On the other hand, there would seem to be reason for some regulation in so far as a high level of pulmonary capillary blood pressure might be harmful on account of the risk of pulmonary oedema.

The existence and rôle of pulmonary vasomotor nerves, which might convey such regulatory influences, has been the subject of numerous investigations, beginning with BRADFORD and DEAN (1889), FRANÇOIS-FRANCK (1895), HENRIQUES (1892), TIGERSTEDT (1903), and others, and more recently by DALY and his co-workers (BERRY and DALY, 1931, DALY and EULER, 1932).

In order to obtain reliable figures for the pulmonary arterial blood pressure, it would seem necessary to keep the animal under as physiological conditions as possible concerning the respiratory functions, and this would involve measurements during spontaneous respiration and a closed thorax. Experiments of this kind are comparatively few. DALY (1937) and later KATZ

and STEINITZ (1940) measured the pulmonary arterial blood pressure by the use of the London technique. In several cases, however, the blood pressure in the right ventricle in animals with a closed thorax has been measured.

The earlier experiments concerning the pulmonary circulation have mostly been undertaken with open thorax, though care has been taken in many instances to avoid pneumothorax and to retain spontaneous respiration. Of course, this does not ensure natural conditions with regard to the intrathoracic pressure, since the mediastinal wall is not a rigid structure as is the thorax. However, many of the results add valuable information as to the response of the pulmonary vessels to nervous and chemical stimuli. LICHTHEIM reported, as early as 1876, that at least  $3/4$  of the total vascular bed of the pulmonary arteries could be excluded without causing any change in the systemic blood pressure. The experiments were performed on curarised dogs, but in one instance also on a non-anaesthetized, spontaneously breathing rabbit with a unilateral pneumothorax. LICHTHEIM also observed a rise in the pulmonary blood pressure of about 50 per cent on compression of the left pulmonary artery, and concluded that the right heart was forcing the same volume of blood through the expanded remaining vessels as through the whole lungs before compression. Vasomotor regulatory influences acting on the systemic circulation were disregarded as a possible cause of the maintenance of the blood pressure level, since there was no sign of even a temporary change in the systemic blood pressure on compression of a pulmonary artery. These results were confirmed by TIGERSTEDT (1903) who stated that compression of the hilus of one lung did not cause any change in the systemic blood pressure. The pulmonary arterial pressure was not measured in his experiments, but the maximal pressure in the right ventricle was only slightly raised or changed, and he therefore advanced the opinion that previously excluded parts of the pulmonary vascular bed were switched into the circulation on compression of the vessels to one lung.

One of the important observations by TIGERSTEDT prompted the statement that no definite relation could be found between the systemic and the pulmonary arterial blood pressure. The relation showed variations between 1: 1.3 and 1: 1.3 in the rabbit. Generally the absolute pressure varied between 11 and 25 mm Hg, though as low a value as 8 mm was found at a systemic pressure

of about 100 mm Hg. The maximal pressure observed on occlusion of the aorta was in no case higher than 51 mm Hg.

The effect of stimulation of the pulmonary vasomotor nerves has been studied by a number of investigators, and more recently by DALY and his co-worker on the dog's isolated perfused lungs. They found that vagal stimulation generally caused a moderate vasodilatation, and stimulation of the stellate ganglion vasoconstriction. On the isolated perfused rabbit's lung EULER (1932) showed that vagal stimulation, similar to injection of acetylcholine, caused a rise in pulmonary arterial pressure, and injection of adrenaline likewise produced a slight rise.

Two questions seem to merit special interest with regard to the hemodynamics of the pulmonary circulation. One is the reaction of the lung vessels to a greatly increased flow, as will occur for instance in muscular work, and the second is in what way the lung vessels react to variations in the blood gases. These problems have been subjected to a study on anaesthetized cats breathing spontaneously with closed thorax or in some cases artificially ventilated.

### Methods.

In all experiments cats weighing 3—4 kg were used, anaesthetized with 0.05—0.06 g chloralose per kg intravenously through a brachial vein. Arterial systemic blood pressure was recorded from one carotid artery.

In order to record the pulmonary arterial pressure the technique of MELLIN (1904) was used in principle, with slight modifications. The thorax was opened widely on the left side between two ribs at the level of the pulmonary artery, and artificial respiration given. By opening the pericardium the pulmonary artery was exposed, care being taken not to sever the nervous tissue between the pulmonary artery and the aorta. The pulmonary artery was compressed near its origin

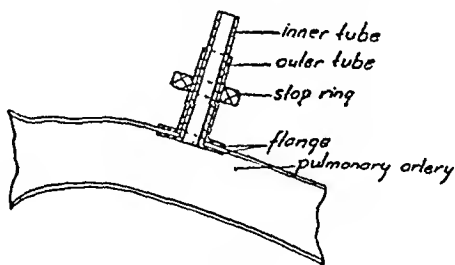


Fig. 1.

by means of a pair of forceps. When completely compressed, a rapid fall in the general blood pressure ensued and a short longitudinal slit was then made in the pulmonary arterial wall by means of fine scissors. The special cannula described below (see Fig. 1) was then quickly inserted and the blood stream through the artery released. The cannula consisted of two well-fitting, concentric metal tubes which could

be moved one within the other. Both were fitted with slightly curved flanges at their ends, giving a good contact surface. When inserting the cannula, the flange of the inner tube was inserted into the lumen of the vessel. Then the outer tube was slid down so that its flange pressed against the flange of the inner tube, thereby gripping the edges of the incision in the vessel wall. The outer tube was fixed in this position by a stop ring. When the cannula was properly inserted, no bleeding occurred. The cannula was then slightly supported in order to prevent compression or kinking of the vessel, and the thorax closed at an inflated state of the lungs. Spontaneous respiration was soon resumed and normal mechanical conditions in the thorax were restored.

For the recording of the pulmonary arterial blood pressure, the cannula was connected with a vertical 30 cm glass tube of the same bore as the cannula (about 3 mm) which was connected to a piston recorder. The actual pressure in mm blood could thus be read at any time and the tracing calibrated at desired intervals. Clotting was prevented by pure heparin, kindly placed at our disposal by Dr. E. JORRES.

As a control, in several instances, the pressure was recorded simultaneously from the left auricle. Various gas mixtures were administered either through a Lovén respiratory valve when the animal was breathing spontaneously, or by attachment of gas bags to the Starling pump.

Muscular work was induced by placing the electrodes of an "Innervator" apparatus, giving alternating current at a frequency of about 50 Hz and with rhythmically varying strength, low on the back and on the lower belly.

Denervation of the sinus region was performed by tying off the tissue between the external and the internal carotids.

## Results.

### 1. Pulmonary Arterial Blood Pressure under Normal Experimental Conditions.

BEUTNER (1852), in Ludwig's laboratory, seems to have made the first observations on the pulmonary blood pressure. In the cat he found values of 7.5—24.7, on an average 17.6 mm Hg. In our preparations, where the spontaneous respiration after closure was satisfactory, but also in those cases where adequate artificial respiration was instituted and the general systemic blood pressure was at an ordinary level, the pulmonary arterial blood pressure was fairly constant. The following table shows the approximate, typical level of the systemic and pulmonary pressures in the 9 experiments where conditions were considered satisfactory.

The relation between the pulmonary and the systemic arterial pressures thus varied between 1: 13—14 and 1: 5, on an average 1: 7—1: 8 during normal steady conditions. This compares well

Nr	Systemic B. P. mm Hg	Pulmonary B. P.		Breathing
		cm water	mm Hg	
1 .....	115	22	16	Artificial
2 .....	150	30	22	Spontaneous
3 .....	160	16	12	"
4 .....	120	20	15	"
5 .....	90	23	17	Artificial
6 .....	120	22	16	Spontaneous
7 .....	150	25	18	"
8 .....	150	23	17	"
9 .....	135	24	18	"
Average:	132	23	17	

with the observations of BEUTNER for cats and of TIGERSTEDT for rabbits where no fixed relation between the pressures was found. The latter author gave the limits for the relative pressures of 1: 1.3 and 1: 1.3, though it should be remarked that the lower ratios corresponded to either unusually high pulmonary pressure or low systemic pressure or both, indicating abnormal conditions.

In the majority of experiments, the pulmonary pressure levels remained quite steady apart from the oscillations produced by the respiratory movements, and this is clearly seen in most of the tracings. In one experiment (nr 4) large waves appeared, however, with a frequency of about 2 in 3 minutes (Fig. 2). The waves had an amplitude of about 5 cm water but in spite of these considerable variations they were hardly reflected at all in the systemic pressure, as seen in fig. 2. This seems to contradict the explanation given by AALKJAER (1935), who considered the

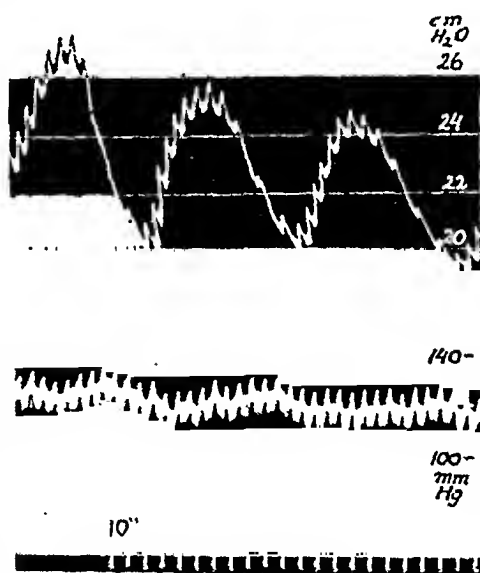


Fig. 2. Cat. 2.9 kg. Chloralose. Upper curve pulmonary arterial pressure with spontaneous waves, lower curve systemic blood pressure. Spontaneous breathing, closed thorax. Time 10 sec.

rhythmical variations in the pulmonary blood pressure to be due to similar variations in the systemic pressure. The waves gradually disappeared when the systemic pressure fell to some 70 mm Hg. It seems reasonable to assume that the effects on both systems are the result of rhythmical activity in the vasomotor centre. Other types of waves occurring independently of the systemic pressure have also been noted (see fig. 3).

Towards the end of an experiment there was mostly a tendency for the pulmonary arterial pressure to rise. Doubtless the reason for this must be found partly in a relative insufficiency of the left heart, resulting in pulmonary congestion, and partly in commencing asphyxial changes in the blood which will increase the pulmonary arterial pressure (see below, p. 309).

## 2. Effect of Variations of Systemic Blood Pressure and Blood Flow on the Pulmonary Arterial Pressure.

### a) Systemic blood pressure changes.

Changes in the systemic arterial pressure were effected in several ways. By clamping the common carotids a marked rise in the general pressure was elicited; this did hardly, however, influence the pulmonary arterial pressure as seen in figure 3. In other cases, a stimulation of the pressoreceptors was brought about by traction of the common carotid and this was followed by the usual fall in systemic blood pressure. Again, this change did not influence the pulmonary arterial pressure to any perceptible degree, nor was this necessarily the case when the blood pressure was raised by asphyxia or vagotomy.

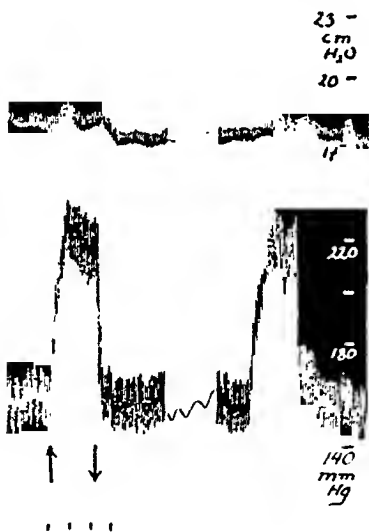


Fig. 3. Cat, 3.5 kg. Chloralose. Upper curve pulmonary arterial pressure, lower curve systemic pressure. Arrows indicate occlusion and opening of common carotid artery. Spontaneous breathing, closed thorax. Time 30 sec.

### b) Muscular work.

The most physiological way of increasing the total blood flow is undoubtedly to induce muscular work. This was done in a num-

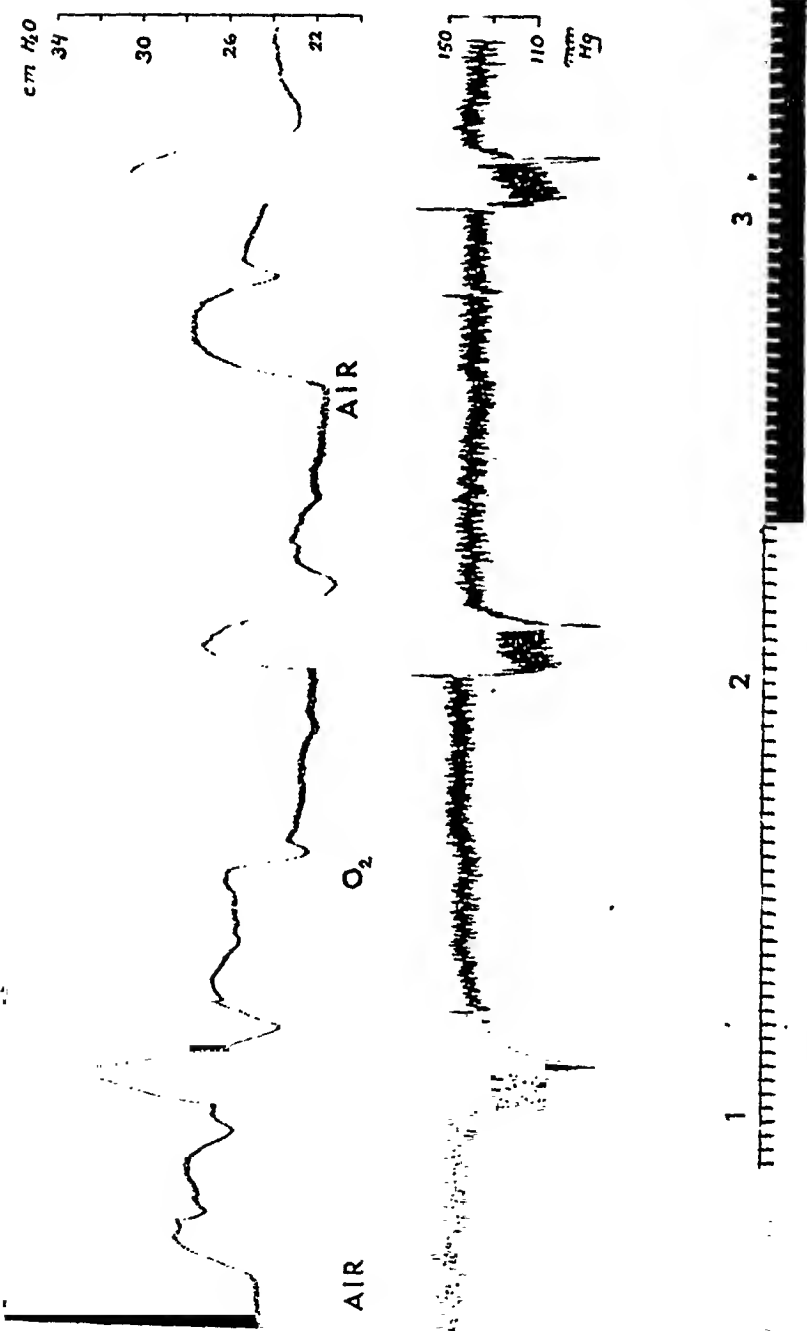


Fig. 4. Cat. 4.1 kg. Chloralose. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. 1. and 3. 1½ minutes moderate muscular work during air breathing. 2. Same during oxygen breathing. Spontaneous breathing, closed thorax. Time 30 sec.



ber of experiments where respiration was spontaneous, and free to respond to the increased demands for gas exchange. In four of the five experiments where muscular work was performed, a definite rise in pulmonary blood pressure ensued, whereas the systemic pressure generally showed a slight fall (Fig. 4). It was also noted that the response was different according to whether the animal was allowed to breathe air or oxygen. This will be discussed further in section 3.

The rise in blood pressure occurred within a few seconds after the commencement of the muscular work, as seen in fig. 4. A partial reason for this quick rise, which continued in the form of a plateau but often declined during the work, is probably a substantial increase in the volume of blood delivered to the right ventricle. It is not surprising that an increase of two or three times the minute volume, as would be the case in the kind of work in question, should be accompanied by a rise of blood pressure, unless a vasodilator action were to take place simultaneously. Whether this action is of reflex type, it is not possible to decide from our experiments. At any rate, a regulatory action could not be effected reflexly through the vagus nerves, since section of these did not alter the response. A reversible fall in pulmonary pressure during muscular work was encountered in one case. It should also be noted that the relative pressure changes in the systemic circulation, where a pressure regulation is known to occur during muscular work (EULER and LILJESTRAND 1946), are as a rule relatively far smaller than those in the pulmonary circulation. This speaks in favour of the assumption that the latter is adapted to a greater flow chiefly by extending the functionally flexible pulmonary vascular bed (of TIGERSTEDT c. p. 274.)

*c) Reduction of pulmonary vascular bed.*

As found by LICHTHEIM and confirmed by TIGERSTEDT occlusion of the arteries to one lung did not cause any change in the systemic blood pressure. The latter author did not even find any rise in the pulmonary arterial pressure which, however, was noted by LICHTHEIM. We have repeated these experiments and have been able to confirm the findings on the first points, as seen in Fig. 5. With regard to the pulmonary arterial pressure, however, this was regularly increased as might be expected, if the same

amount of blood has to pass through the reduced vascular bed. The rise was quite moderate, however, amounting to approximately 20 percent.

d) *Effect of injections into the pulmonary artery.*

Incidentally, it was noted that injection of Ringer's solution into the pulmonary artery through the cannula was followed by a slight fall in pressure, which persisted for a minute or so. The reason for this effect may have been a distension of the vessels which was only slowly overcome. This is also in keeping with the ability of the lung vessels to adjust themselves easily to an increased flow.

### 3. Effects of Inhalation of Gas Mixtures of Varying Oxygen and Carbon Dioxide Content.

The influence of variations in the blood gases on the systemic blood pressure has been subject to numerous observations under varying experimental conditions. The discoveries of HEYMANS and his co-workers (1932) concerning the reflex control of blood pressure through the medium of the chemoreceptors constitute the most important recent contributions to this problem. Recently EULER and LILJESTRAND (1946) have studied the effects of blood gas variations on the systemic blood pressure of the cat and summarized the results.

With regard to the effects of the blood gases on the pulmonary blood pressure, this question may be said to merit special interest on account of the gas exchange in the lungs.

We have tested in repeated experiments the effects of gas mixtures rich and poor in oxygen, as well as rich in carbon dioxide.

a) *Oxygen-lack and pure oxygen.* If the animal is made to breathe a mixture containing 10—11 per cent oxygen in nitrogen, an increase of the pulmonary arterial pressure was invariably

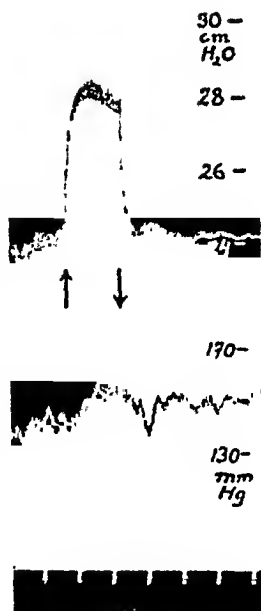


Fig. 5. Cat. 3.0 kg. Chloralose. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. Arrows indicate occlusion and opening of left lung artery. Artificial respiration, open thorax. Time 30 sec.

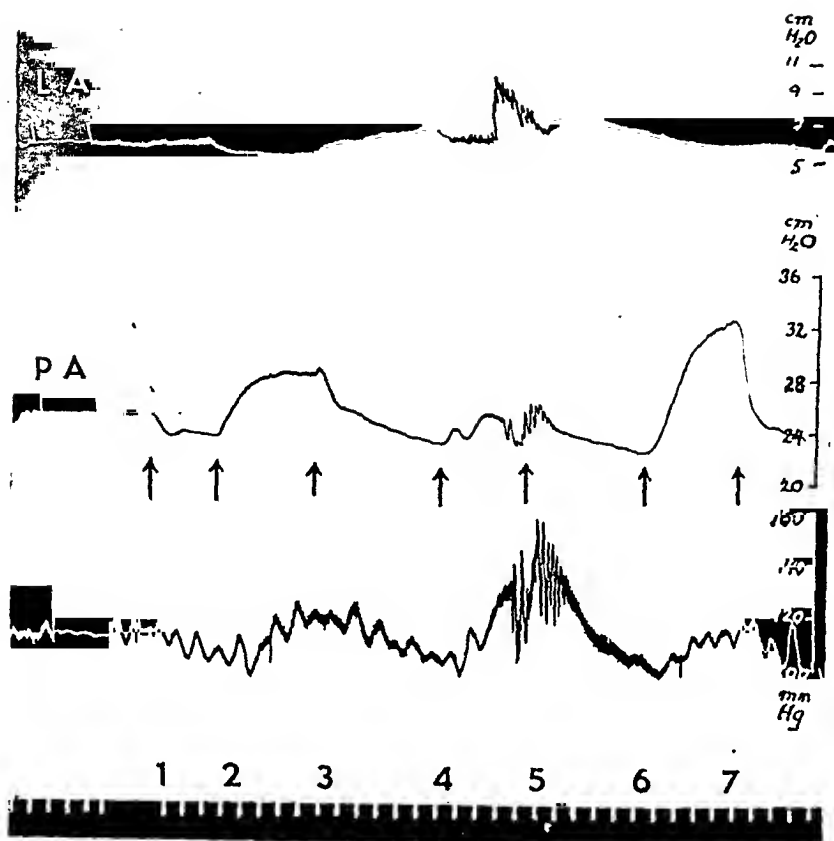


Fig. 6. Cat. 3.9 kg. Chloralose. Uppermost curve pressure in left auricle, middle curve pulmonary arterial pressure, lower curve systemic blood pressure. 1.  $O_2$  (from air). 2. 6.5 p. c.  $CO_2$  in  $O_2$ . 3.  $O_2$ . 4. 18.7 p. c.  $CO_2$  in  $O_2$ . 5.  $O_2$ . 6. 10.5 p. c.  $O_2$  in  $N_2$ . 7.  $O_2$ . Artificial respiration, open thorax. Time 30 sec.

observed. This effect has not been influenced to any perceptible degree by vagotomy or by extirpation of the stellate ganglia, and must be regarded as a direct effect on the lung vessels unless a reflex mechanism of altogether unknown nature comes into play. Also breathing of pure oxygen caused a distinct fall in pulmonary arterial pressure as illustrated in fig. 6. The considerable increase in the minute volume of the heart during moderate muscular work led to a rise in the pulmonary arterial pressure about equal to that observed during rest, when air was inspired instead of oxygen (fig. 4). It must be concluded that this

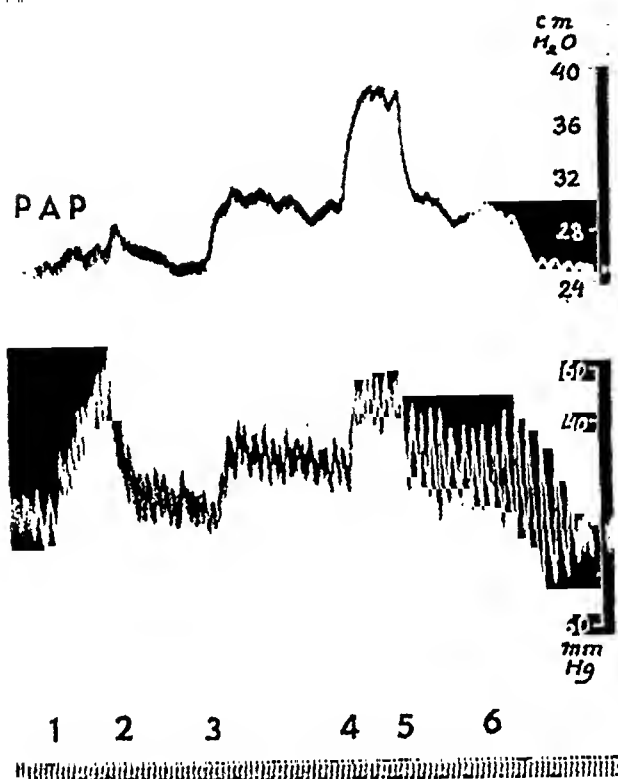


Fig. 7. Cat, 3.5 kg. Chloralose. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. 1. 20.5 p. c.  $\text{CO}_2$  in  $\text{O}_2$  from  $\text{O}_2$ . 2.  $\text{O}_2$ . 3. Air. 4. 10.5 p. c.  $\text{O}_2$  in  $\text{N}_2$ . 5. Air. 6.  $\text{O}_2$ . Spontaneous breathing, closed thorax. Time 10 sec.

latter effect is in the main caused by other factors than an increase of the blood flow.

From our observations it can be inferred that even during normal experimental conditions there will be a certain degree of vasoconstriction in the lung arteries caused by the desaturation of the venous blood. Fig. 4 illustrates that this effect is added to that of muscular work, indicating different mechanisms in the two cases.

b. *Carbon dioxide*. When the gas inhaled by the experimental animal was changed from oxygen to a mixture of 6.5 per cent carbon dioxide in oxygen, a moderate increase in pulmonary arterial blood pressure ensued. Higher concentrations of carbon

dioxide were also tested with the same result. In some of these experiments, the carbon dioxide gradually increased since the animal was breathing pure oxygen from a spirometer with no means for the absorption of the carbon dioxide. The rise in pulmonary blood pressure was, however, relatively moderate and less than the increase in systemic pressure. Thus in one experiment the latter increased from 134 mm Hg at an alveolar carbon dioxide of 8.67 per cent to 186 mm at 19.80 per cent alveolar carbon dioxide *i. e.* 40 per cent, whereas the pulmonary pressure only increased from 17.2 cm blood to about 20 cm or 16 per cent. The effect of oxygen lack occurred independently of section of the vagodepressors or removal of the stellate ganglia and seems to be a direct action on the vessels. A similar kind of direct action of the blood gas content, though acting conversely, is shown by the placental arteries, which contract vigorously under the action of oxygenated blood (SCHMITT 1922). Also the effect of carbon dioxide noticeably differs from that generally found. The higher percentages were not, however, accompanied by a rise in the left auricular pressure and the lower concentrations (6.5 p. *e.* CO<sub>2</sub> in O<sub>2</sub>) could even cause a slight lowering of the pressure in the left auricle (Fig. 6). During asphyxia the marked rise in pulmonary blood pressure might occur with only a minute rise in the left auricular pressure (from 3 to 4 cm blood). A comparison of the effects of various blood gases on the systemic as well as the pulmonary blood pressure is found in fig. 7.

In the experiments on the effect of muscular work on the pulmonary circulation, it was noted that the rise in blood pressure was definitely greater when the animal was breathing air than during oxygen inhalation. This is obviously due to an increased degree of desaturation of the venous blood during muscular work in the former case.

#### 4. Effects of Various Drugs on the Pulmonary Arterial Pressure.

In previous investigations the effect of drugs on the pulmonary blood pressure has been studied repeatedly. Thus MELLIN (1904) tested the effect of, among others, nicotine, heart glucosides, amyl nitrite, nitroglycerine and adrenaline. Nicotine caused a slight fall followed by a small rise, and adrenaline in doses of 12—20  $\mu$ g only a moderate rise of 2—3.5 mm Hg or 15—25 per cent. Higher doses (30—40  $\mu$ g) had a still smaller action.

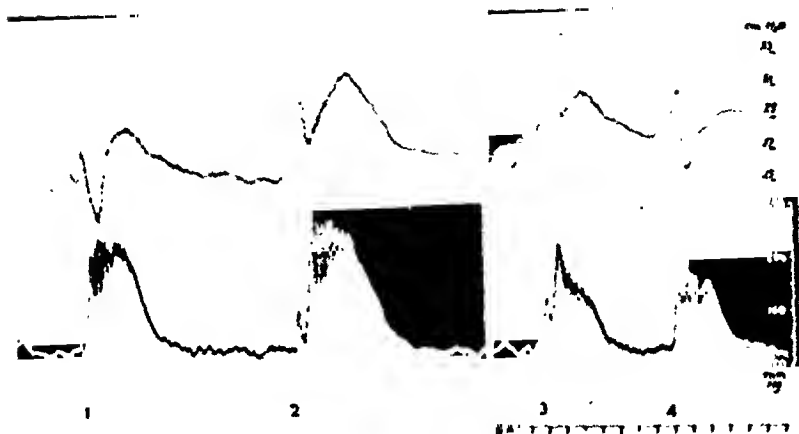


Fig. 8. Cat. 3.0 kg. Chloralose. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. 1.  $5 \mu\text{g}$  dl-nor-adrenaline hydrochloride. 2.  $5 \mu\text{g}$  l-adrenaline intravenous injections. 3.  $2.5 \mu\text{g}$  l-adrenaline. 4.  $2.5 \mu\text{g}$  dl-nor-adrenaline hydrochloride injected into the pulmonary artery. Spontaneous breathing, closed thorax. Time 30 sec.

DALY and co-workers have tested the effect of various drugs on the dog's pulmonary circulation and found a rise after adrenaline and a fall after acetylcholine.

#### a) *Adrenaline.*

The effects of adrenaline have been somewhat varying. In our best preparations, however, a definite and considerable rise in pressure has been observed. Thus in expt 9, intravenous injections of  $5 \mu\text{g}$  adrenaline caused a rise of 7 cm blood or from 25 to 32 cm (28 per cent). Direct injection of  $2.5 \mu\text{g}$  adrenaline into the pulmonary artery gave a rise of 6 cm from 27 to 33 cm. The curve in the former case showed a typical step as illustrated by fig. 8 and the rise had about the same duration as in the systemic blood pressure, though the wellknown "step" in this case occurred earlier. On injection of the smaller dose directly into the pulmonary circulation the primary rise was rather prominent as compared with the second slower rise.

In other experiments the effects have been less conspicuous, however, and in one case no effect whatever was noted after a dose of 0.1 mg which caused a very marked effect on the systemic pressure. In expt 4 the only response to  $5 \mu\text{g}$  adrenaline was a slight fall in pressure.

In conclusion it may be said that adrenaline, if effective at all on the pulmonary arterial blood pressure, causes small effects only, as compared with those on the systemic circulation.

b) *Arterenol*.

In recent investigations by EULER (1946) it has been demonstrated that extracts of most organs and adrenergic nerves contain a sympathomimetic substance which conforms in its biological and chemical action with nor-adrenaline or arterenol. It was therefore considered of interest to compare the action of this substance with adrenaline. On the systemic blood pressure the effects were of about the same strength, weight for weight, though the adrenaline "step" failed to occur with the arterenol. The action on the pulmonary blood pressure revealed certain differences in action, however. Thus the total rise was markedly less with arterenol and a definite fall was noted between a primary short rise and a second prolonged one. (Fig. 8.) The same picture was obtained in principle when a smaller dose, 2.5  $\mu\text{g}$ , was injected directly into the pulmonary artery through the cannula. Since it may be assumed that stimulation of the adrenergic nerves to the lung will liberate a substance with the same type of action as arterenol, this result is of significance for the evaluation of the results of stimulation of the pulmonary vasomotor nerves of adrenergic origin (cf. below, section 5).

A similar action was obtained with dihydroxy-nor-ephedrine as with arterenol.

c) *Veritol*.

This substance, in a dose of 2 mg, caused a very conspicuous and long-lasting rise in the pulmonary arterial blood pressure from 27 to 35 cm blood (30 per cent).

d) *Acetylcholine*.

In doses of 1—5  $\mu\text{g}$  acetylcholine caused a fall in pulmonary arterial pressure. Even the smallest dose tried, 1  $\mu\text{g}$ , may produce a fall from 27 to 21 cm blood (22 per cent) (Fig. 9). On the other hand 10  $\mu\text{g}$  had no effect in one case. This effect of acetylcholine agrees with the effect in dogs, but is in contrast to the effect observed on the perfused lung of the rabbit, where vasoconstriction and a rise in pressure were found (EULER 1932).

It will also be noted that the reaction to acetylcholine, here as in the rabbit, is parallel to the effect of vagal stimulation.

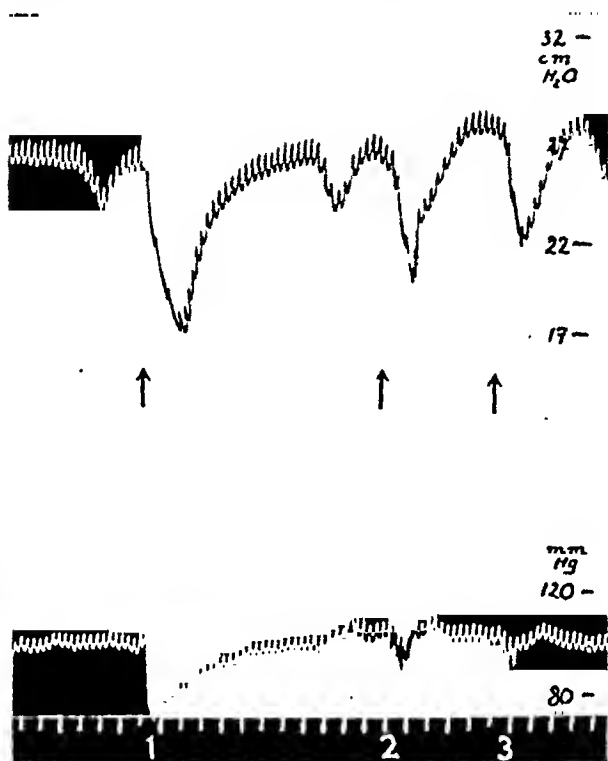


Fig. 9. Cat, 2.9 kg. Chloralose. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. 1.  $1 \mu\text{g}$  acetylcholine. 2. Weak stimulation of right vagosympathetic nerve in the neck. 3. Same. Spontaneous breathing, closed thorax. Time 10 sec.

e) *Histamine*.

Histamine in a dose of  $2 \mu\text{g}$  caused a moderate fall in pressure in one experiment.

f) *Vasopressin*.

This substance in a dose of 1—2 units caused a lasting but moderate increase of pressure.

g) *Coramine*.

50 mg coramine, which caused a rise of the systemic blood pressure from 75 to 135 mm Hg, had no definite action on the pulmonary pressure.





blood expelled by the right ventricle is driven through the same organ, and for one common purpose, constitutes the paramount difference between the lesser circulation and the systemic blood flow. This also leads to different demands with regard to the pressure regulation within the two systems. The systemic blood pressure in the aorta and its main branches must be kept within rather narrow limits even at very great alterations in the general blood flow, and at the same time the distribution of the circulating blood must be regulated according to the greatly varying needs of the different organs. This is made possible by the cooperation of local factors inducing a dilatation of the arterics of the working organs, and an adaptation of the general tone of the arterial system to the prevailing conditions. As is well known, the tone exercised by the vasomotor centre is in its turn controlled by impulses from the baro- and chemoreceptors of the carotid and aortic regions. The function of the pressure regulation for the pulmonary artery also seems to serve the purpose of ensuring the necessary blood flow through the different parts of the lungs and of preventing too great a rise of the pressure. An increase of the blood flow is accompanied by a rise of the pulmonary arterial blood pressure, as is clearly illustrated by the results obtained after occlusion of the vessels to one lung. But, whereas in this experiment the blood flow is approximately doubled, the relative increase of the pressure is only about 20 per cent. This demonstrates the great distensibility of the pulmonary vascular bed. The observation mentioned above, that the small extra pressure caused by injection of a few ml of Ringer's solution in the pulmonary artery was followed by a definite and somewhat lasting fall of the pulmonary pressure, may also illustrate this distensibility. It will consequently be expected that an adaptation to muscular work will be possible with only a moderate rise of the pulmonary arterial blood pressure. This corresponds well with our results. The degree of oxygen unsaturation of the mixed venous blood (and its carbon dioxide tension) will also be of significance for the level attained. Since this factor already comes into play during ordinary rest, and to a greater extent during muscular work, the increase of pulmonary arterial pressure caused by the augmentation of the blood flow alone should be smaller than the one actually observed in our experiments.

It is also required, however, that the blood becomes distributed to the different parts of the lungs in such a way, that the

alveolar air will give off oxygen and take up carbon dioxide fairly evenly throughout the lungs. Mechanical factors, *e. g.* variations in posture, will easily influence this distribution (cp. RICH and FOLLIS 1942). If the blood flow becomes inadequate in relation to the ventilation in some parts of the lungs, the corresponding alveolar air will become richer in oxygen and poorer in carbon dioxide than the rest of the lungs. But this will lead to a dilatation of the blood vessels of that part of the lungs with a redistribution of the blood as a consequence. It is interesting to note that oxygen want and carbon dioxide accumulation have exactly the reverse local effects on the vessels of the systemic and pulmonary circulations respectively; in both cases, however, they seem to be adapted for their special purposes. They cause a dilatation of the vessels of the working organs which need a greater blood supply than during rest, but they call forth a contraction of the lung vessels, thereby increasing the blood flow to better aerated lung areas, which leads to improved conditions for the utilization of the alveolar air. This will also imply that oxygen breathing during muscular work will facilitate the blood flow through the pulmonary vascular bed and allow a greater minute volume at a lower pressure, *i. e.* with less strain on the right heart.

We have concluded from our experiments that the increased pulmonary arterial pressure during oxygen-want is due to a direct action on the pulmonary vessels — in contrast to the corresponding effect on the systemic blood pressure which is produced through the stimulation of the chemoreceptors, whereas the direct action on the vasomotor centre itself is depression. Accumulation of carbon dioxide acts in the same direction as oxygen-want, though less pronouncedly. Probably the site of action is in the vessels themselves, as is the case with the placental vessels.

The only evidence we have obtained of a nervous control of the pulmonary blood pressure consists of the rhythmical variations observed. It is also characteristic that the mechanisms described enable a regulation which seems to be relatively independent of the simultaneous regulation of the systemic pressure. It is quite possible, however, that a certain connection exists between a nervous control of the pulmonary arterial pressure and the corresponding regulation of the systemic blood pressure, as indicated by the results obtained by SCHWIEGK (1935).

### Summary.

The pulmonary arterial blood pressure was recorded in anaesthetized cats by means of a special cannula, according to MELLIN's technique. In most experiments the thorax was closed and the animal was breathing spontaneously.

The pulmonary arterial pressure in 9 experiments averaged 23 cm water, or approximately 17 mm Hg, at an average systemic pressure of 132 mm Hg. The average ratio thus was about 1: 8, with the limits 1: 5 and 1: 14.

Pressure variations of 1—2 cm blood synchronous with the breathing were regularly recorded. In one case, slow large waves of 1—2 minutes duration and about 5 cm amplitude were observed.

Even great variations in the systemic blood pressure, elicited from the pressoregulating reflex mechanisms, were hardly accompanied by variations in the pulmonary arterial pressure.

During muscular work a moderate rise in pulmonary blood pressure generally occurred, greater when air was breathed than when oxygen alone was administered.

Clamping the pulmonary artery to one lung did not cause any change in systemic pressure (confirming LICHTHEIM and TIGERSTEDT) but caused a moderate rise in pulmonary arterial pressure.

Breathing of pure oxygen lowered the pulmonary arterial pressure and oxygen-lack raised it. Carbon dioxide 6.5—20.5 per cent in oxygen raised the pressure slightly, but constantly. These effects were not influenced by vagotomy.

The effect of injections of adrenaline, nor-adrenaline, acetylcholine and histamine and of stimulation of pulmonary nerves were studied in some cases.

The experiments seem to warrant the conclusion, that the regulation of the pulmonary blood flow is mainly mediated by a local action of the blood and alveolar gases leading to an adequate distribution of the blood through the various parts of the lungs according to the efficiency of aeration.

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# **The Hydrodynamics of Semi-Permeable Tubes** with Reference to the Glomerular Function of the Kidney.

By

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and FLEMMING RAASCHOU.

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In the course of investigations into the function of the kidney with the subject in the erect posture we were able (see previous publications BRUN, KNUDSEN, and RAASCHOU) by means of the inulin and diodrast clearances to make certain observations on variations in the renal function. The interpretations of these observations have necessitated an endeavour to clarify the hydraulic laws governing the flow of colloidal fluids through tubes through whose walls proceeds a filtration of the water phase alone, and the laws applying to this filtration.

Clarification of these laws would be of importance, not only to our understanding of the flow of fluids through the kidney but to all problems involved in the passage of water between the capillaries and the surrounding tissues.

In the above investigations the glomerular filtration was determined as the inulin clearance and the blood flow in the kidneys as the diodrast clearance, and a calculation was made of their mutual proportions; this value indicates the proportion between the quantity of liquid filtered through the glomeruli and the volume of blood flowing through the kidneys. It is called the

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This investigation was performed with the support of Miss P. A. Brandt's Bequest.

filtration fraction and is expressed as a percentage of the blood flow.

Below we shall make a brief recapitulation of the results of these investigations.

When the experimental person was tilted from the horizontal to the passive erect posture, we found a distinct fall in the blood flow and a somewhat less marked fall in the glomerular filtration whereby the filtration fraction rose. However, simultaneously, with these changes in the renal function we found a number of changes in the composition and pressure of the blood, changes which made it difficult to interpret the variation in the clearances.

We observed the following changes in the pressure and composition of the blood: 1) Usually an increase of the mean blood pressure, 2) an increase of the plasma-protein concentration and with it an increase of the colloid osmotic pressure, 3) an increase in the cell volume of the blood. Furthermore, the viscosity of the blood rises as a consequence both of the higher protein concentration and of the higher cell volume.

Two possibilities suggest themselves in explanation of these changes in filtration, blood flow and filtration fraction: 1) They may be due *solely* to the observed changes in the blood pressure and composition, or 2) they are *also* due to changes in the calibre of vas afferens and/or vas efferens.

These variations in the blood pressure and composition may, when the values of the two are in suitable ratio, cause changes in the blood flow values in the direction indicated by the results of the experiments, but in the present stage of our knowledge we cannot determine the total effect of these various factors.

Consequently, it was necessary to decide upon some means of forming an idea of the effects of these factors. This was not all plain sailing, as in this instance POISEUILLES' law, which is considered applicable to the flow in the minor vessels of the organism, cannot be applied to tubes generally; the law was both discovered and subsequently theoretically derived for laminary flow through *impermeable* capillary tubes (POISEUILLE 1847). In our case we are concerned with tubes that are highly *permeable*, for about 20 per cent. of the inflow volume has left the capillary bed by the time it reaches the outflow. It is impossible to say anything *a priori* about the flow itself in such permeable tubes.

As far as we can see from the available literature, the discussion on glomerular-hydrodynamic problems has never touched

upon this particular aspect of the glomerular vessels. Strictly speaking, this is only natural, as it has not previously been possible to operate with such well-defined values of the blood flow as is expressed by the diodrast clearance.

### Formulation of the Hydraulic Problem.

We had now to tackle the problem of deducing the physical laws governing the laminar flow of colloidal solutions in semi-permeable tubes, which would enable us to calculate the pressure and flow at any point in the tube. Knowing the flow value at a given point one can calculate the filtration up to that point by subtracting that flow from the flow volume at the inflow.

These problems lay beyond the physical and mathematical horizon of the medical collaborators in this work, and therefore we approached Professor A. E. BRETTING, who is professor of hydraulics at the Royal Technical College. He very obligingly and with great interest undertook to solve this purely hydraulic problem. At the same time we learned that it had seemingly not previously been dealt with in hydraulic literature.

### Deduction of the Hydraulic Laws for the Flow of Colloidal Fluids in Semi-Permeable Tubes.

Fig. 1 is a schematic drawing of a semi-permeable tube having the radius  $r$  and the length  $L$ . Above the tube are pressure and fluid-flow curves.

It is assumed that the fluid consists of a water phase of specific gravity  $\gamma_0$  containing protein in the colloidal state. The sp. gravity of the protein is put at  $\gamma_A$ . As the water phase alone permeates the wall of the tube, the protein concentration will vary at the different points on the tube. The sp. gravity of the solution at any point is indicated by  $\gamma$ .

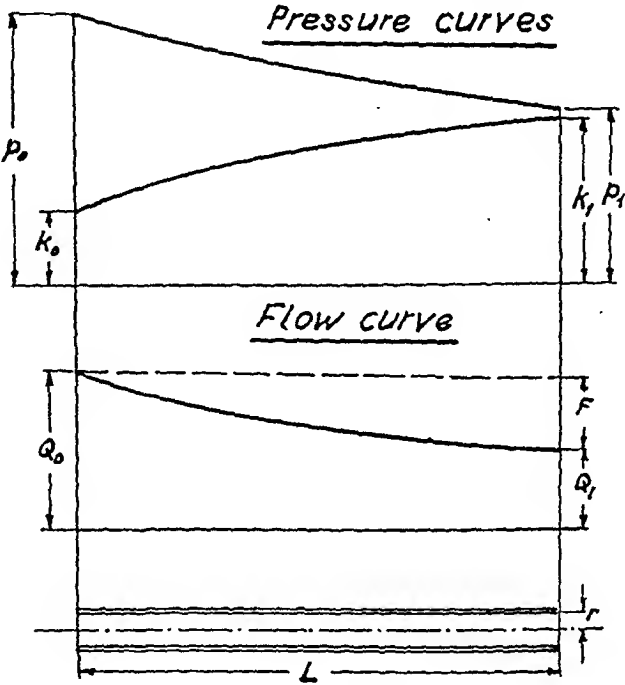
As the water alone can pass through the tube wall, there arises a colloid osmotic pressure which reduces the effective filtration pressure. This colloid osmotic pressure is called  $k$ . It appears from works by VERNEY (1926) and KROGH and NAKAZAWA (1927) as well as from our own investigations, that with a falling protein concentration the colloid osmotic pressure falls with it, but to a lesser degree. Thus, even if one cannot positively count upon direct proportionality between protein concentration and colloid osmotic pressure, one may undoubtedly within certain limits presuppose a rectilinear variation of the colloid osmotic pressure with the protein concentration, so that within these limits we may put:

$$k = k' + a \cdot m$$



where  $\alpha$  is the protein concentration, here determined by the volume, and  $k'$  and  $m$  are constants of the same dimensions as  $k$ .

If the absolute volume of protein flowing into the tube per second



- $p$  = Hydraulic pressure.
- $k$  = Colloid osmotic pressure.
- $Q$  = Flow of fluid.
- $F$  = Filtrated volume
- $r$  = Radius of the tube
- $L$  = Length of the tube

Fig. 1.

is designated by  $a$ , this volume accordingly is constant throughout the length of the tube. The total flow at any given point being  $Q$ , the concentration here is:

$$\alpha = \frac{a}{Q} \dots \dots \dots (1)$$

At the inflow the total fluid flow is  $Q_0$ . The concentration at the inflow is therefore:

$$c_0 = \frac{n}{Q_0} \quad \dots \dots \dots (1a)$$

When the concentration  $c_0$  and  $Q_0$  are known,  $n$  is then also known.

We put the relative fluid flow in proportion to the flow at the inflow as  $q$ . Thus when  $Q$  represents the flow at a given point we get:

$$q = \frac{Q}{Q_0} \quad \dots \dots \dots (2)$$

At the inflow:

$$q = 1 \quad \dots \dots \dots (2a)$$

As already stated, the colloid osmotic pressure  $k$  is calculated:

$$k = k' \cdot c \cdot m \cdot v \quad \dots \dots \dots (3)$$

If  $k_0$  designates the colloid osmotic pressure at the inflow, we get from formulas (1), (1a) and (3):

$$k = k_0 \cdot \frac{c}{c_0} = \frac{k_0}{q} \cdot \frac{k_0}{q} \quad \dots \dots \dots (3a)$$

The variation in the hydraulic pressure  $p$  determines the flow through the tube. The difference between the hydraulic and the colloid osmotic pressures ( $p - k$ ) determines the filtration through the tube wall.

Assuming for practical reasons that the pressures are reduced by  $k'_0$ , the relative hydraulic pressure  $\gamma$  (in proportion to the pressure  $p_0$  at the inflow) is expressed by:

$$\gamma = \frac{p - k'}{p_0 - k'_0} \quad \dots \dots \dots (4)$$

At the inflow:

$$\gamma_0 = 1 \quad \dots \dots \dots (4a)$$

The relative colloid osmotic pressure  $z$ :

$$z = \frac{k - k'}{p_0 - k'_0} \quad \dots \dots \dots (5)$$

If we insert (3a) into (5) we get

$$z = \frac{k_0 - k'}{q} \cdot \frac{1}{p_0 - k'_0} = \frac{z_0}{q} \quad \dots \dots \dots (5a)$$

where  $z_0$  is the relative colloid osmotic pressure at the inflow.

For the sp. gravity of the solution at a given point we find that:

$$\gamma = \frac{n}{Q} \gamma_0 + \frac{(Q - n)}{Q} \gamma_0 = \gamma_0 \left( 1 - \frac{n}{Q} \left( 1 - \frac{\gamma_0}{\gamma_0} \right) \right) = \gamma_0 \left( 1 - a \left( 1 - \frac{\gamma_0}{\gamma_0} \right) \right) \quad (6)$$

We now write:

$$\mu = a \left( 1 - \frac{\gamma_0}{\gamma_0} \right) = \frac{\gamma_0 - \gamma}{\gamma_0}$$

and thus get:

$$\gamma = \gamma_0 (1 - \mu) \quad \dots \dots \dots (6a)$$

We now consider an elementary part of the tube of the length  $dl$ . The fall (I) on the energy grade line may here be reckoned as being identical with the fall on the line of hydraulic pressure, the velocity heights being insignificantly small.

$$I = -\frac{1}{\gamma} \frac{dp}{dl} \quad \dots \dots \dots (7)$$

and  $l$  being the abscissa for a given point of the tube,  $r$  the tube radius and  $\nu$  the kinematic viscosity of the solution, which is assumed to be constant, for the laminary flow in the element (Poiseulles' law) we get

$$Q = -\frac{\pi \cdot g \cdot r^4}{8\nu} \cdot \frac{1}{\gamma} \frac{dp}{dl}$$

$g$  = the acceleration of the gravity.

We now introduce a relative abscissa, assuming that:

$$x = \frac{l}{L} \quad \dots \dots \dots (8)$$

where  $L$  is the total length of the tube.

With (4), (6) and (8) we then get:

$$q = \frac{Q}{Q_0} = -\frac{\pi \cdot g \cdot r^4 (p_0 - k')}{8\nu Q_0 \cdot L} \cdot \frac{1}{\gamma_b (1 - \mu)} \frac{dy}{dx} \quad \dots \dots (8a)$$

With the help of (1) and (1a),  $\mu$  may be rewritten as:

$$\mu = \frac{\mu_0}{q}, \text{ where } \mu_0 \text{ signifies}$$

$\mu_0 = \frac{\gamma_b - \gamma_0}{\gamma_b}$ , where  $\gamma_0$  is the sp. gravity of the solution at the inflow.

We now find:

$$q - \mu_0 = -\frac{\pi \cdot g \cdot r^4 (p_0 - k')}{8\nu \gamma_b Q_0 \cdot L} \frac{dy}{dx} \quad \dots \dots \dots (9)$$

If we insert:

$$A = \frac{8\nu \gamma_b Q_0 L}{\pi g \cdot r^4 (p_0 - k')} \quad \dots \dots \dots (10)$$

where  $A$  is a constant in a certain problem, we obtain:

$$q - \mu_0 = -\frac{1}{A} \frac{dy}{dx} \quad \dots \dots \dots (9a)$$

On differentiating (9a),  $\mu_0$  being constant, we get:

$$\frac{dq}{dx} = -\frac{1}{A} \frac{d^2y}{dx^2} \quad \dots \dots \dots (11)$$

The filtration is reckoned as being proportional to  $(p - k)$  and to the particular area of the tube wall on the length element  $dl$ . We write:

$$dQ = -c \frac{p - k}{\gamma_b} \cdot 2\pi r dl$$

Here  $c$  is a constant, proportional to the coefficient of permeability of the material of the tube wall and inversely proportional to the wall thickness. We now presuppose that the thickness of the wall is great in proportion to the diameter of the pores, so that the acceleration pressures may be ignored.

With (2), (4), (5) and (8) we get:

$$\frac{dq}{dx} = -\frac{c}{Q_0} (p_0 - k') \cdot L \cdot \frac{2\pi r}{\gamma_0} (y - z) \quad \dots \quad (12)$$

With (10), (11) and (12) we get:

$$\frac{d^2y}{dx^2} = B(y - z) \quad \dots \quad (13)$$

where

$$B = \frac{16 \pi c L^2}{g \cdot r^3} \quad \dots \quad (14)$$

is a constant. From (5a) and (9a) we get:

$$z = \frac{z_0}{q} = \frac{z_0}{\mu_0 - \frac{1}{A} \frac{dy}{dx}} \quad \dots \quad (15)$$

On inserting (15) into (13) we get:

$$\frac{d^2y}{dx^2} = B \left( y - \frac{z_0}{\mu_0 - \frac{1}{A} \frac{dy}{dx}} \right) \quad \dots \quad (16)$$

which is the differential equation for the flow of colloidal solutions in semi-permeable tubes. If we put  $z_0 = 0$  we get:

$$\frac{d^2y}{dx^2} = B \cdot y \quad \dots \quad (16a)$$

which is the differential equation for the flow of non-colloidal fluids in semi-permeable tubes.

Equation (16) can easily be rewritten:

$$\mu_0 \cdot A \frac{d\left(\frac{dy}{dx}\right)}{dx} - \frac{1}{2} \frac{d\left(\left(\frac{dy}{dx}\right)^2\right)}{dx} = B \left( \mu_0 \cdot A \cdot y - \frac{1}{2} \frac{dy^2}{dx} - A \cdot z_0 \right) \quad \dots \quad (16b)$$

On the right side of this equation the first member is very small in proportion to the others. Therefore with approximation we replace  $\int_0^x y dx$  (= the area between the pressure line and the axis of  $x$  limited by the ordinates  $x = 0$  and  $x = x$ ) with  $\frac{1}{2} (1 + y)x$ , which corresponds to rectilinear variation of the pressure line on the said distance. It is

then easy to integrate the equation. By this means we obtain a 2nd degree equation for determining  $\frac{dy}{dx}$  and find:

$$\frac{dy}{dx} = A \left( \mu_0 - \sqrt{1 - B \left( \frac{1 - y^2}{A^2} - x \frac{2z_0 - \mu_0(1 + y)}{A} \right)} \right) \quad (17)$$

Expressed as a proportion of  $Q_0$  the total filtration for the whole length of the tube is equal to the filtration fraction  $f$ . We have:

$$f = \frac{Q_0 - Q_1}{Q_0} = 1 - q_1 \quad (18)$$

where  $q_1$  corresponds to  $x = 1$ .

From (9a) we get for  $x = 1$

$$q_1 = \mu_0 - \frac{1}{A} \left( \frac{dy}{dx} \right)_{x=1} \quad (19)$$

From (17) we get  $\left( \frac{dy}{dx} \right)_{x=1}$  to which corresponds  $y = y_1$ ;

$$\left( \frac{dy}{dx} \right)_{x=1} = A \left( \mu_0 - \sqrt{1 - B \left( \frac{1 - y_1^2}{A^2} - \frac{2z_0 - \mu_0(1 + y_1)}{A} \right)} \right) \quad (20)$$

Then from (19) and (20) we get:

$$q_1 = \sqrt{1 - B \left( \frac{1 - y_1^2}{A^2} - \frac{2z_0 - \mu_0(1 + y_1)}{A} \right)} \quad (21)$$

For known pressures and known inlet flow the filtration fraction can be determined by (21) and (18).

In addition to this  $q_1$  formula we shall also set up a formula for  $y$ , the relative pressure. This can be done by integrating (17) by means of a development of functions.

We insert:

$$y = 1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \dots \quad (22)$$

from which we get:

$$\frac{dy}{dx} = \beta_1 + 2\beta_2 x + 3\beta_3 x^2 + \dots \quad (23)$$

If we insert (22) and (23) into (17) and collect the coefficients thus:  $x^0, x, x^2, x^3 \dots$ , and if we assume that each of these expressions is equal to 0, we arrive at formulae for the determination of  $\beta_1, \beta_2, \beta_3, \dots$ . On making this calculation we get:

$$\begin{aligned} \beta_1 &= -a_1 A & \beta_5 &= a_5 \frac{B^4}{A^3} + b_5 \frac{B^3}{A} + c_5 A B^2 \\ \beta_2 &= a_2 B & \beta_6 &= a_6 \frac{B^5}{A^4} + b_6 \frac{B^4}{A^2} + c_6 B^3 \\ \beta_3 &= -a_3 \frac{B^2}{A} - b_3 A \cdot B & & \\ \beta_4 &= -a_4 \frac{B^3}{A^2} + b_4 B^2 & & \end{aligned} \quad (24)$$

where:

$$\begin{aligned}
 a_1 &= 1 - \mu_0 & a_2 &= \frac{1}{10} z_1 (1 - z_1) \left( -\frac{1}{2} + \frac{5}{3} z_1 - \frac{5}{4} z_1^2 \right) \\
 a_2 &= \frac{1}{2} (1 - z_1) & b_1 &= \frac{1}{120} z_1 (1 - 5z_1) + \frac{\mu_0^2}{240} (3 - 21z_1 + 20z_1^2) \\
 a_3 &= \frac{1}{6} z_1 (1 - z_1) & c_1 &= \frac{1}{120} (1 - \mu_0)^2 \\
 b_2 &= \frac{1}{6} (1 - \mu_0) & a_4 &= \frac{1}{6} (10 a_1 a_2 + 12 a_1 a_3 - a_2) \\
 a_4 &= \frac{1}{8} z_1 (1 - z_1) \left( \frac{2}{3} - z_1 \right) & b_2 &= \frac{1}{6} \left( 10 a_1 b_1 + 12 a_1 b_2 - 12 a_1 b_3 - b_1 \right. \\
 b_4 &= \frac{1}{24} \left( 1 + \frac{1}{2} \mu_0 (1 - 3z_1) \right) & & \left. - a_1 a_4 + a_1 a_3 - \frac{1}{2} \mu_0^2 a_1 \right) \\
 & & c_4 &= \frac{1}{6} \left( 10 a_1 c_1 - 12 b_1 b_4 - c_2 + a_1 b_4 + \right. \\
 & & & \left. + a_1 b_3 + \frac{1}{2} \mu_0 b_4 \right)
 \end{aligned} \tag{25}$$

Inserting (24) into (22) we get:

$$y = 1 - a_1 A x + a_1 B x^2 + \left( -a_2 \frac{B^2}{A} - b_2 A B \right) x^3 + \dots \tag{26}$$

If we put  $x = 1$ , we get  $y = y_1$ , and thus find the following expression for  $y_1$ :

$$\begin{aligned}
 y_1 &= \left( 1 - a_1 A \right) + \left( a_2 - b_1 A \right) B + \left( -a_2 \frac{1}{A} + b_4 + c_1 A \right) B^2 + \left( -a_4 \frac{1}{A^2} \right. \\
 &\quad \left. + b_2 \frac{1}{A} + c_4 + \dots \right) B^3 + \left( a_2 \frac{1}{A^2} + b_4 \frac{1}{A^2} + \dots \right) B^4 + \left( a_4 \frac{1}{A^4} + \dots \right) \\
 &\quad B^5 + \dots \tag{27}
 \end{aligned}$$

We may now be faced with the problem of determining the constants  $A$  and  $B$  which correspond to certain values of  $y_1$  and  $q_1$ . It is imaginable that the data determining  $A$  and  $B$  cannot be measured direct. This problem is solved by means of the formulae (27) and (21). From (27) we determine correlative values of  $A$ ,  $B$  and  $y_1$ . Aided by for example a graphic illustration we find various sets of values ( $A$ ,  $B$ ) corresponding to the measures  $y_1$ . These sets of values can now be inserted into (21) until this equation gives the measured value of  $q_1$ ; we have then found the correct values of  $A$  and  $B$ .

If the fluid flowing through the tube is a non-colloidal solution (water

for instance), it is the differential equation (16 a) that must be solved. Its complete solution is:

$$y = C_1 \operatorname{Cos} x \sqrt{B} + C_2 \cdot \operatorname{Sin} x \sqrt{B}^1$$

where  $C_1$  and  $C_2$  are arbitrary constants.

If we insert the marginal conditions:

for  $x = 0$  is  $y = 1$  and for  $x = 1$  is  $y = y_1$ , we get:

$C_1 = 1$  and  $C_2 = \frac{y_1 - \operatorname{Cos} \sqrt{B}}{\operatorname{Sin} \sqrt{B}}$  which after some little converting gives:

$$y = \frac{\operatorname{Sin} (1-x) \sqrt{B} + y_1 \operatorname{Sin} x \sqrt{B}}{\operatorname{Sin} \sqrt{B}} \dots \dots \dots (28)$$

Formula (9a) becomes for  $\alpha = 0$ :

$$q = -\frac{1}{A} \frac{dy}{dx} \dots \dots \dots (29)$$

From (28) we get:

$$\frac{dy}{dx} = \frac{\sqrt{B}}{\operatorname{Sin} \sqrt{B}} (-\operatorname{Cos} (1-x) \sqrt{B} + y_1 \operatorname{Cos} x \sqrt{B})$$

which, inserted into (29), gives:

$$q = \frac{\sqrt{B}}{A} \frac{\operatorname{Cos} (1-x) \sqrt{B} - y_1 \operatorname{Cos} x \sqrt{B}}{\operatorname{Sin} \sqrt{B}}$$

$\frac{\sqrt{B}}{A}$  is determined by the fact that  $x = 0$  must give  $q = 1$ ; thus:

$$\frac{\sqrt{B}}{A} = \frac{\operatorname{Sin} \sqrt{B}}{\operatorname{Cos} \sqrt{B} - y_1} \dots \dots \dots (30)$$

which on being inserted gives:

$$q = \frac{\operatorname{Cos} (1-x) \sqrt{B} - y_1 \operatorname{Cos} x \sqrt{B}}{\operatorname{Cos} \sqrt{B} - y_1} \dots \dots \dots (31)$$

For  $x = 1$  we get  $q = q_1$ , thus:

$$q_1 = \frac{1 - y_1 \operatorname{Cos} \sqrt{B}}{\operatorname{Cos} \sqrt{B} - y_1} \dots \dots \dots (32)$$

With the aid of formulae (28), (31) and (32) it is possible to clarify pressure and flow completely.

When  $y_1$  and  $q_1$  have been determined by experiment, (32) may be employed for finding the value of  $B$ .  $B$  being known,  $A$  may be determined by (30). It is often possible to find all the values composing

<sup>1</sup>  $\operatorname{Cos}$  and  $\operatorname{Sin}$  are here employed as expressions for the corresponding hyperbolic functions.

A by means of direct measurement; in that case we have an excellent means of checking the correctness of the formulae, in that A determined by (30) and by direct measurement must agree.

Thus we have now deduced the following formulae:

1) *Colloidal solutions.*

$$q_1 = \sqrt{1 - B \left( \frac{1 - y_1^2}{A^2} - \frac{2z_0 - \mu_0(1 + y_1)}{A} \right)}$$

$$y = 1 + \beta_1 x + \beta_2 x^2 + \beta_3 x_3 + \dots$$

where  $\beta$  is determined according to formulae (24) and (25).

2) *Non-colloidal solutions*

$$q_1 = \frac{1 - y_1 \cos \sqrt{B}}{\cos \sqrt{B} - y_1}$$

$$y = \frac{\sin(1 - x) \sqrt{B} + y_1 \sin x \sqrt{B}}{\sin \sqrt{B}}$$

In these formulae:

$$q = \frac{Q}{Q_0}; \quad y = \frac{p - k'}{p_0 - k'}; \quad z_0 = \frac{k_0 - k'}{p_0 - k'}; \quad x = \frac{l}{L}; \quad \mu_0 = \frac{\gamma_b - \gamma_0}{\gamma_b}$$

$$A = \frac{8\nu\gamma_b Q_0 \cdot L}{\pi \cdot g \cdot r^4 (p_0 - k')} \quad \text{and} \quad B = \frac{16\nu \cdot c \cdot L^2}{g \cdot r^3}, \quad \text{where}$$

$Q_0$  = the flow at the entrance

$Q$  = the flow at a given point in the tube with abscissa  $l$

$p_0$  = the hydraulic pressure at the entrance

$p$  = the hydraulic pressure at a given point with abscissa  $l$

$k_0$  = the colloid osmotic pressure at the entrance

$L$  = the length of the tube

$r$  = the radius of the tube

$c$  = the coefficient of permeability of tube wall

$\gamma_b$  = the specific gravity of the water phase

$k'$  = a constant, its significance being shown by formula (3).

$\gamma_0$  = the sp. gravity of the solution at the entrance.

$\nu$  = the mean value of the kinematic viscosity:

It will be appropriate here to mention the prerequisites under which the formulae were deduced.

1) It is assumed that the protein concentration and the colloid osmotic pressure are inter-dependent as:  $k = k' + am$ .

2) The flow is assumed to be laminar, i. e. Reynold's number must be lower than 2300. (REYNOLD'S number:  $v \times 2 \times r/\nu$  (Reynolds, 1883), where  $v$  = velocity.



3) The kinematic viscosity  $\left(\frac{g \cdot \eta}{\gamma}\right)$  where  $g$  = the acceleration of gravity,  $\eta$  = the ordinary viscosity and  $\gamma$  = the sp. gravity of the fluid) is assumed to be constant throughout the tube. If this pre-requisite is not entirely satisfied, we can with good approximation reckon with a mean value.

4) The thickness of the tube wall is assumed to be great in proportion to the diameter of the pores, so that the acceleration pressures may here be ignored.

5) The tube wall must be of such a nature that the coefficient of permeability  $c$  for the wall material is constant, and the diameter of the tube must also be constant.

6) Structure viscosity means that the viscosity of a fluid changes with the pressure. In the present work it is not assumed that this is of any importance at the pressures likely to be met with (BOESEN 1945).

### Model Experiments.

We have now endeavoured to prove the validity of these laws by means of model experiments.

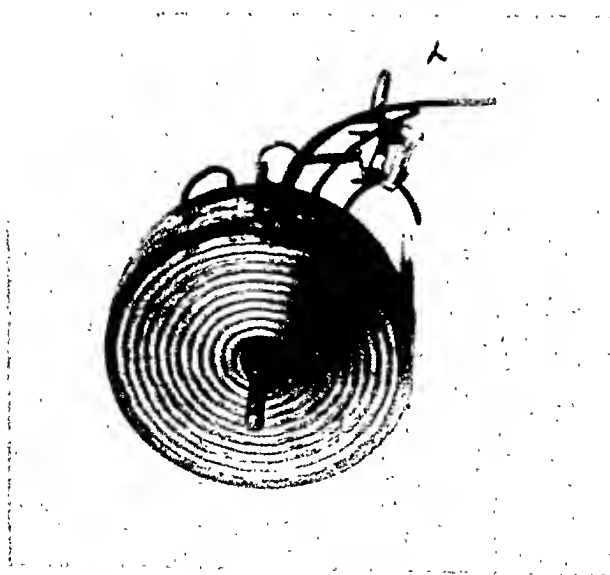
Professor REHBERG very kindly placed an apparatus at our disposal (fig. 2) which was suitable for our purpose.

The apparatus was constructed by Professor A. KROGH in 1918 for dialysis experiments. It consists of four circular tin plates having a diameter of 1450 mm. On both sides of each plate is a spiral groove 1.48 mm deep and 3.95 mm wide. The plates are assembled with semi-permeable membranes between, thus forming two long spiral-shaped channels separated by the membranes and each having a total length of 10 m when the channels in the various layers are connected one with the other. The cross-section of the channels is rectangular. Each plate-system is connected by means of short rubber tubes, so that the filtrate or the fluid from which filtration proceeds may flow on into the following systems. The total length of the channel of each plate is 250 cm, and the filtering area is 100 cm<sup>2</sup>. The total area of the filtering surfaces is 400 cm<sup>2</sup>.

*Arrangement:* The fluid flows to the apparatus under constant pressure. The inflow and outflow pressures are measured by means of open standpipes. The outflow pressure is regulated independently of the inflow pressure by an overflow device. The volume



Fig. 2.



of flow is measured in a finely graded burette, and the filtered volume in a horizontal capillary pipette.

*Membranes:* For the experiments with *water* we employed membranes of factory-made parchment paper.

For those with *plasma* we used collodium membranes; these were prepared by pouring 30 cc collodium solution (3 % collodium in ether-alcohol (1: 7)) into a metal ring (diameter 1640 mm) lying on a horizontal, plane-ground glass plate. After allowing 8 minutes for drying the membrane was covered with distilled water, after which it was easy to disengage from the glass. This membrane is completely impermeable to protein.

*Correction of our formulae:* Our formulae were all deduced for a circular cross section, but it is easy to correct them according to BOUSSINESQUE so that they become valid for the rectangular cross section of the channels in the model (BOUSSINESQUE 1932). The only changes made are A and B, which receive the values:

$$A = \frac{12\nu\gamma_b Q_0 \cdot L}{bh^3 g \cdot (p_0 - k')} \frac{1}{1 - \frac{192}{\pi^5} \left(\frac{h}{b}\right) \left\{ \text{Tg} \left(\frac{\pi b}{2 h}\right) + \frac{1}{3^5} \text{Tg} \left(\frac{3\pi b}{2 h}\right) + \dots \right\}} \quad (33)$$

$$B = \frac{12 \cdot \nu c \cdot L^2}{g \cdot h^3} \frac{1}{1 - \frac{192}{\pi^5} \left(\frac{h}{b}\right) \left\{ \text{Tg} \left(\frac{\pi b}{2 h}\right) + \frac{1}{3^5} \text{Tg} \left(\frac{3\pi b}{2 h}\right) + \dots \right\}} \quad (34)$$

Here *b* and *h* respectively mean the width and height of the rectangular cross section of the channel. It will be seen that A can be re-written

$$A = \Theta \left( \frac{\nu}{\nu_{20}} \right) \frac{Q_0}{p_0 - k'} \quad (33a)$$

$$\text{where } \Theta = \frac{12 \nu_{20} \gamma_b \cdot L}{bh^3 \cdot g} \frac{1}{1 - \frac{192}{\pi^5} \left(\frac{h}{b}\right) \left\{ \text{Tg} \left(\frac{\pi b}{2 h}\right) + \frac{1}{3^5} \text{Tg} \left(\frac{3\pi b}{2 h}\right) + \dots \right\}} \quad (35)$$

and where  $\nu_{20}$  expresses the kinematic viscosity of water at 20° C.

We have made sure that the flow in the model is laminar, for Reynold's number reaches a maximum of about 20 in our experiments. The course adopted for the experiments is this: First a number of tests are made with *water*, and from these we determine A and B. A is found from the formula (33a). From formula (35) we see that  $\Theta$  is an absolute constant, depending only on the dimensions of the model and the sp. gravity of the water phase. This constant can now be determined direct by measuring  $\gamma_c$ , *L*, *b* and *h*, and it can be found from the test results by means of formula (30). The two values of  $\Theta$  thus arrived at must agree.

After the water tests we carry out a series with *plasma*. From the results we determine  $B$  by the formula (21), which is re-written to:

$$B = \frac{1 - q_1^2}{\frac{1 - y_1^2}{A^2} - \frac{2z_0 - \mu_0(1 + y_1)}{A}} \dots \dots \dots (36)$$

For  $A$  we introduce here the expression (33a) with the constant  $\theta$  determined by the water tests. It will now appear that  $\frac{B}{r}$  is a constant,  $\frac{B}{r_{20}^0}$

at any rate within the individual test series.

Within the limit of protein concentrations in which our experiments were made, calculations have shown that without committing any error of importance we may put  $k' = 0$ . This also applies to the experiment described on page 341.

### The water tests.

We made 13 tests with water at a temperature of about 20° C. Of these, 8 had an inflow pressure ( $p_0$ ) about 1310 mm fluid column (FC), the remaining having  $p_0 =$  about 790 mm fluid column.

Measures were taken of the pressures at the inflow and outflow ( $p_0$  and  $p_1$ ), the quantity of filtering water  $Q_0 - Q_1$  and the flow at the outlet ( $Q_1$ ).

The results were as follows:

Test No.	$p_0$ mm FC	$p_1$ mm FC	$y_1$	$Q_0$ cc/Min	$Q_1$ cc/Min	$f$	$q_1$
1	1312.5	1297.5	0.990	0.637	0.477	0.252	0.748
2	1312.5	1294.5	0.987	0.882	0.726	0.177	0.823
3	1312.5	1291.0	0.985	1.055	0.904	0.144	0.856
4	1312.5	1290.0	0.983	1.000	0.848	0.152	0.848
5	1312.0	1270.0	0.968	1.825	1.676	0.082	0.918
6	1310.0	1230.0	0.938	3.72	3.57	0.0403	0.960
7	1302.0	1110.0	0.852	9.58	9.44	0.0156	0.984
8	1296.0	1018.0	0.786	12.95	12.80	0.0113	0.989
9	792.5	770.5	0.971	0.727	0.634	0.128	0.872
10	792.5	766.0	0.966	1.271	1.147	0.0970	0.903
11	792.0	755.0	0.952	1.525	1.430	0.0630	0.937
12	789.0	709.0	0.894	3.685	3.60	0.0245	0.976
13	781.0	624.0	0.787	7.61	7.52	0.0118	0.988

According to the theory we have (32):  $q_1 = 1 - f = \frac{1 - y_1 \cos \sqrt{B}}{\cos \sqrt{B} - y_1}$

As will be seen,  $q_1$  alone is a function of  $y_1$  if  $B$  is constant. This is the case when the coefficient of permeability of the filter  $c$  and the kinematic viscosity  $\gamma$  (the temperature) are constant.

We have drawn  $f\%$  (the filtration fraction) as a function of  $y_1$  (fig. 3). According to formula (32) the same function is calculated for various values of  $B$ . It has turned out that  $B = 0.00276$  ( $\cos \sqrt{B} = 1.00138$ ) agrees well with the results of the tests. It will be seen from the curve that the filtration fraction rises very steeply as  $y_1$  approaches 1. When  $y_1$  is in the vicinity of 1, the pressure determining the amount of filtration ( $Q_0 - Q_1$ ) is very nearly constant throughout the length of the tube, i. e. for all tests in which  $y_1$  is near 1 the amount of filtration is fairly constant. However, it is

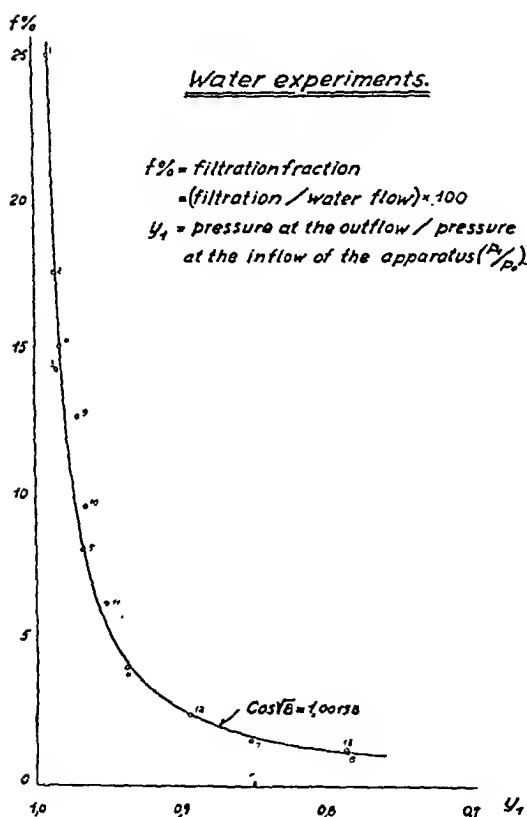


Fig. 3.

the pressure difference that determines the flow itself, and this difference may vary within wide limits, whereby the flow is subject to considerable variation. From this it follows that

the filtration fraction,  $f\% = \frac{Q_0 - Q_1}{Q_0} \times 100$  will vary considerably when  $y_1$  is near 1. When  $y_1$  is somewhat farther away from 1 both filtration amount and flow will vary, so that the

filtration fraction decreases slowly with falling  $y_1$ , as the curve shows. The tests thus give:

$$B = 0.00276.$$

Proceeding now to examine the value of  $A$ , making use of formulae (30) and (33a). According to (30) we find ( $B$  being small in proportion to 1, and as we can write  $\sin \sqrt{B} \cong \sqrt{B}$ ):

$$A = \cos \sqrt{B} - y_1 \quad \dots \dots \dots (37)$$

If this formula is compared with formula (33a) we find:

$$\Theta = \frac{p_0}{Q_0} \frac{r_{20}}{r'} (\cos \sqrt{B} - y_1) \quad \dots \dots \dots (38)$$

From this formula  $\Theta$  can be determined solely from the calculated value of  $B$  and the measured pressures and flows. From formula (35)  $\Theta$  can be determined solely from the measurement of  $\gamma_b$  and the dimensions of the model. As we require to introduce  $Q_0$  in cc/min. and  $p_0$  in mm fluid column in the two formulae, we must replace the factor 12 in formula (35) with 2.

On calculating according to formula (38) (the water temperature in all the tests being about 20°) we find:

Test No.	$10^3 \frac{Q_0}{p_0} \frac{r_{20}}{r'}$	$y_1$	$A$	$\Theta$
1	0.485	0.990	0.011	22.6
2	0.671	0.987	0.014	20.9
3	0.805	0.985	0.016	19.9
4	0.762	0.983	0.018	23.6
5	1.39	0.968	0.083	23.7
6	2.84	0.938	0.068	22.2
7	7.25	0.852	0.149	20.6
8	10.00	0.786	0.215	21.5
9	0.917	0.971	0.030	(32.7)
10	1.608	0.966	0.035	21.8
11	1.926	0.952	0.049	(25.4)
12	4.67	0.894	0.107	22.9
13	9.74	0.787	0.214	22.0

When  $\Theta$  is determined direct according to formula (35) we find the following, reckoning

$$\nu_{20} = 1.00 \cdot 10^{-2} \text{ sq. cm/sec.}$$

$$h = 0.1475 \text{ cm}$$

$$b = 0.395 \text{ cm}$$

$$L = 1000 \text{ cm}$$

$$\gamma_b = 1.000 \text{ g/cc}$$

$$g = 981 \text{ cm/sec}^2$$

$$\Theta = \frac{2 \cdot 1.00 \cdot 10^{-2} \cdot 1.000 \cdot 1.000}{0.395 \cdot 0.1475^3 \cdot 981}$$

$$\frac{1}{1 - \frac{192}{\pi^5} \frac{0.1475}{0.395} \left\{ Tg \left( \frac{\pi}{2} \cdot \frac{0.395}{0.1475} \right) + \frac{1}{3^5} Tg \left( \frac{3\pi}{2} \cdot \frac{0.395}{0.1475} \right) + \dots \right\}} = 21.1$$

The above table shows that the mean value of  $\Theta$  is 22.0. The mutual deviation between the two values of  $\Theta$  is easily explained; if the temperature of the water had been  $19.5^\circ$  for example, agreement would have been complete. An alteration of 0.22 mm in  $h$  would also have given the same result. Thus it must be said that the experiments with water provide handsome confirmation of the validity of the laws.

The result was thus:

$$B = 0.00276$$

$$A = 22.0 \times \frac{\nu}{\nu_{20}} \times \frac{Q_o}{p_o - k'}$$

### Plasma tests.

Here four series of experiments were made, I, II, III and IV, with human serum with a protein concentration of 7 % in I, II and III and 4.29 % in IV.

The colloid osmotic pressure at the inflow (in  $Q_o$ ) was measured according to KROGH (1936) and KROGH and NAKAZAWA (1st method (1927)). In tests I, II and III it was found to be 354 mm fluid column and in test IV: 144 mm fluid column.

The viscosity of  $Q_o$  was measured by the method described by W. R. HESS. In tests I, II and III it was found to be 1.9 in relation to water at  $26^\circ \text{C}$ , and in test IV: 1.5 in relation to water at  $26^\circ \text{C}$ .

$\gamma_b$  and  $\gamma_o$  were determined by weighing serum and protein-free ultra-filtrate of serum in the pycnometer at 26° C. The following results were obtained:

Test	$\gamma_b$	$\gamma_o$
I, II, III	1.005	1.028
IV	1.005	1.018

The results of the tests are tabularized below.

As will be seen,  $\frac{B}{\nu_{20}}$  in the test groups I, II and IV are very nearly

constant, whereas for group III it is a somewhat lower value. Possibly this may be due to the error in measuring the colloid osmotic pressure having most influence on test III, in which the hydraulic pressure is lowest (see formula 36).

Therefore, in our opinion the conclusion to be drawn from our model experiments with water and plasma is that the flow in our model proceeds according to the deduced formulae.

Test No.	$P_0$ mm FC	$P_i$ mm FC	$Q_1$ cc/Min	F cc/Min	$Q_0$ cc/Min	B	$\frac{B}{\nu_{20}}$
I 1	1463	1369	4.25	0.778	5.028	0.0119	0.0072
2	1458	1112	9.06	0.358	9.368	0.0113	0.0068
II 1	1463	1412	2.18	0.640	2.820	0.0098	0.0059
2	1462	1377	3.97	0.602	4.572	0.0098	0.0059
3	1461	1298	4.10	0.800	4.400	0.0104	0.0063
4	1459	1159	7.80	0.298	8.098	0.0111	0.0067
5	1455	955	13.00	0.275	13.275	0.0115	0.0070
III 1	703	689	1.16	0.886	1.496	0.0076	0.0046
2	702	671	2.18	0.289	2.889	0.0076	0.0046
3	701	641	2.80	0.151	2.951	0.0071	0.0043
4	699	556	7.07	0.181	7.201	0.0075	0.0046
IV 1	707	674	3.23	0.434	3.664	0.0080	0.0062
2	707	654	4.87	0.438	5.308	0.0073	0.0057
3	706	596	6.68	0.286	6.966	0.0072	0.0056
4	704	427	16.50	0.288	16.788	0.0073	0.0057
5	707	673	2.99	0.473	3.463	0.0078	0.0060



For tests I, II and III:	For test IV:
$A = 22.00 \cdot \frac{r'}{r'_{20}} \frac{Q_0}{p_0} = 36.4 \frac{Q_0}{p_0}$	$A = 22.0 \frac{r'}{r'_{20}} \frac{Q_0}{p_0} = 28.8 \frac{Q_0}{p_0}$
$\mu_0 = \frac{1.005 - 1.028}{1.005} = -0.023$	$\mu_0 = \frac{1.005 - 1.018}{1.005} = -0.013$
$z_0 = \frac{354}{p_0}$	$z_0 = \frac{149}{p_0}$
$B = \frac{1 - q_1^2}{\frac{1 - y_1^2}{A^2} - \frac{2z_0 - \mu_0(1 + y_1)}{A}}$	

All tests carried out at 26° C.

### Application of the laws to glomerular circulation.

We shall now endeavour to apply to the glomerular circulation in the kidney the laws deduced above for the flow of fluids in semi-permeable tubes. Naturally, in transferring results obtained from experiments with a simple physical model to a circulatory system so complicated as the flow of the blood through the kidney, the outcome of one's calculations must be judged with great caution.

First, an account must be given of the *presuppositions* which had to be taken into account in order to be able to make the necessary calculations, and in this we must primarily refer to the presuppositions mentioned on page 331 as being necessary to our deduction of the laws. At this present stage we presuppose that the permeability is independent of any dilation of the glomerular capillaries; even if dilation should occur, LANDIS's (1927) investigations of the filtration through frog capillaries show that we must assume that normal dilated capillaries are not more permeable than contracted capillaries.

The hydraulics of the kidney are further complicated by the fact that the circulating fluid contains a suspension of corpuscular elements, the erythrocytes. From an hydraulic point of view, however, the laws of flow are not generally altered by the intermixture of such corpuscular elements. As FÄHRÆUS and LINDQUIST (1931) have demonstrated, when blood flows through tubes

of narrow calibre the blood corpuscles move along the axis of the tube, whereas there is a purely plasma layer out at the tube wall. One may say that actually the flow proceeds in a "plasma tube". According to KROGH's observations (1936), this axial flow occurs in a capillary if only the diameter is greater than the diameter of the erythrocytes. As the diameter of the glomerular capillary is twice that of the erythrocyte diameter (according to VIMTRUP), there are good reasons for supposing that the flow in the former is axial. This being so, we consider it justifiable to apply the deduced formulae, provided that we take into consideration the influence of the cellular elements on viscosity and specific gravity.

In our calculations we assume that the capsular pressure in the Bowmann capsule remains constant on both horizontal and erect postures. The capsular pressure depends partly in the filtration, partly on the efflux through the tubules and therefore should perhaps be corrected for changes in the filtration; this, however, we have not done.

It is also presupposed that the radius of the glomerular capillaries is the same in the horizontal as in the erect posture.

Furthermore, it is presupposed that the number of glomeruli functioning is the same in the two postures.

In the course of our model experiments with plasma we observed that the permeability of the collodium membrane decreases at the commencement of a test and only gradually becomes constant, a phenomenon which must be interpreted as the gradual choking up of membrane pores with protein. The natural assumption is that there are similar variations in the obstruction of the pores of the glomeruli, which represents another complication in the evaluation of the function of glomerular capillaries.

Here we shall quote a concrete experiment from one of our earlier publications (1945). The individual was first placed in the horizontal posture, in which the inulin clearance was 122 cc/min. and the diodrast clearance 572 cc/min. The plasma-protein concentration was 7.91 %. He was then tilted to a passive erect posture of  $+40^\circ$ , when the inulin clearance fell to 84 cc/min. and the diodrast clearance to 320 cc/min., whereas the plasma-protein concentration rose to 8.49 %. There was no change in the blood pressure. The viscosity of total blood rose from 28.9 sec. to 30.8 sec. The specific gravity of whole blood was determined with the pycnometer at 1.058 ( $\gamma_0$ ), whereas that of the water

phase was determined on a protein-free ultrafiltrate at 1.010 ( $\gamma_b$ ).

We shall now try whether, with the aid of the new laws and our experimental results, anything can be deduced regarding pressures and the manner in which these are altered in glomeruli in the erect posture. Our task is to decide whether the observed changes in the pressure and composition of the blood alone can explain the observed decreases in filtration and renal plasma flow, or whether we must also assume that changes take place in the diameters of the inflow and outflow.

First, *the horizontal posture*.

All the terms being the same as in the foregoing, the following values can be given:

Measured values:

$$\gamma_0 = 1.058 \text{ g/cc}$$

$$\gamma_b = 1.010 \text{ g/cc}$$

$$Q_0 = 572 \text{ cc/min.} = \text{diodrast clearance.}$$

$$Q_1 = 450 \text{ cc/min.} = \text{diodrast clearance} - \text{inulin clearance.}$$

Before we can make the calculations we must also know the hydraulic and colloid osmotic pressures. These are not measured; according to current theories  $p_0$  is put at 60 mm Hg and  $k_0$  at 24 mm Hg. We reckon with a capsular pressure  $p_c$ , which we put at 12 mm Hg. This capsular pressure diminished all the effective pressures by its value. In conformity with a work by SMITH, CHASIS, GOLDRING and RANGES (1940), we furthermore assume that in the horizontal posture there is pressure balance at the end of the capillary, i. e. we assume  $p_1 - p_c = k_1$ . Whether this assumption is correct or not we cannot say positively, but we shall revert to the question later. We now have:

Estimated values:

$$p_0 = 60 \text{ mm Hg.}$$

$$p_c = 12 \text{ mm Hg.}$$

$$k_0 = 24 \text{ mm Hg.}$$

$$p_1 - p_c = k_1.$$

We are now able to calculate the following values:

$$\mu_0 = \frac{\gamma_b - \gamma_0}{\gamma_b} = \frac{1.010 - 1.058}{1.010} = -0.0475$$

$$z_0 = \frac{k_0}{p_0 - p_c} = \frac{24}{60 - 12} = 0.500$$

$$q_1 = \frac{Q_1}{Q_0} = \frac{450}{572} = 0.787$$

$$f = 1 - q_1 = 0.213$$

$$y_1 = z_1 = \frac{z_0}{q_1} = \frac{0.500}{0.787} = 0.635$$

From the formula (25) we can calculate the coefficients  $a$ ,  $b$ ,  $c$  . . . according to which it is possible to calculate  $A$  and  $B$  by the method described on page 9. We find:

$$\begin{array}{ll} a_1 = 1.0475 & a_5 = 0.000521 \\ a_2 = 0.25 & b_5 = 0.00674 \\ a_3 = 0.0417 & c_5 = -0.00914 \\ b_3 = 0.1746 & a_6 = 0.000565 \\ a_4 = 0.00521 & b_6 = 0.000845 \\ b_4 = 0.0422 & c_6 = -0.00272 \end{array}$$

According to formula (27) we have:

$$\begin{aligned} y_1 = 0.635 = & \left(1 - 1.0475 A\right) + \left(0.25 - 0.1746 A\right) B + \left(-0.0417 \frac{1}{A} + \right. \\ & \left. + 0.0422 - 0.00914 A\right) B^2 + \left(-0.00521 \frac{1}{A^2} + 0.00675 \frac{1}{A} - 0.00272\right) B^3 + \\ & + \left(0.000521 \frac{1}{A^3} + 0.000845 \frac{1}{A^2}\right) B^4 + \left(0.000565 \frac{1}{A^4}\right) B^5 + \dots \end{aligned}$$

and, according to formula (21), as:

$$\begin{aligned} 1 - y_1^2 &= 1 - 0.635^2 = 0.597 \\ 2z_0 - \mu_0(1 + y_1) &= 2 \cdot 0.5 + 0.0475(1 + 0.635) = 1.078 \\ q_1 = 0.787 &= \sqrt{1 - B \left( \frac{0.597}{A^2} - \frac{1.078}{A} \right)} \end{aligned}$$

From these two equations with the two unknown  $A$  and  $B$  we now find that:

$$A = 0.405 \quad B = 0.390$$

On introducing these values  $A$  and  $B$  into equation (26) we find:

$$p = 48(1 - 0.424x + 0.0975x^2 - 0.0432x^3 + 0.00453x^4 + 0.000605x^5 + 0.000147x^6 \dots) \text{ mm Hg}$$

As  $q = \mu_0 - \frac{1}{A} \frac{dy}{dx}$  we find:

$$Q = 572(1 - 0.481x + 0.32x^2 - 0.0423x^3 - 0.007x^4 - 0.002x^5 + \dots) \text{ cc/min.}$$

As  $z = \frac{z_0}{q}$ , the colloid osmotic pressure may now be calculated:

$$k = \frac{24}{1 - 0.481x + 0.320x^2 - 0.0423x^3 - 0.007x^4 - 0.002x^5 + \dots} \text{ mm Hg.}$$

Curves representing capsular pressure, colloid osmotic pressure and hydraulic pressure are plotted in fig. 4 in continuous lines.

We now proceed to examine the *passive erect posture* and what changes this alteration in the posture may bring about in the pressures. Here we have observed certain changes in  $v$ ,  $Q_0$  and  $\gamma_0$  as well as in the protein

Pressure curves of a single glomerular capillary in the horizontal posture (—) and in the passive erect posture (+60°)(-----).

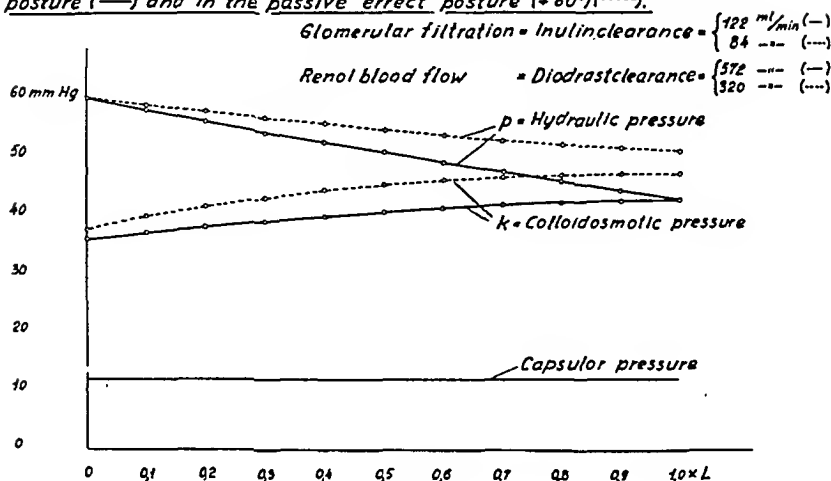


Fig. 4.

concentration, and through these in the colloid osmotic pressure. We now provide all the expressions associated with the horizontal posture with the index  $l$  and the standing posture with  $s$ . We find:

$$\begin{aligned} \frac{v_s}{v_l} &= 1.062 & \frac{k_{os}}{k_{ol}} &= 1.0735 \\ \frac{p_{os}}{p_{ol}} &= \frac{60}{60} = 1.00 & \frac{\gamma_{os}}{\gamma_{ol}} &= \frac{1.062}{1.058} = 1.025 \\ \frac{Q_{os}}{Q_{ol}} &= \frac{320}{572} = 0.560 \end{aligned}$$

On calculating for the horizontal posture we found:

$$A_l = 0.405 \quad B_l = 0.390$$

Recollecting the formulae (10) and (14) we may now write:

$$A_s = A_l \left( \frac{v_s}{v_l} \right) \left( \frac{Q_{os}}{Q_{ol}} \right) \left( \frac{p_{ol}}{p_{os}} \right)$$

$$B_s = B_l \left( \frac{v_s}{v_l} \right)$$

$\left( \frac{r_l}{r_s} \right)$  being reckoned as being equal to 1. Thus we find:

$$A_s = 0.405 \cdot 1.062 \cdot 0.560 \cdot \frac{1}{1.00} = 0.241$$

$$B_s = 0.390 \cdot 1.062 = 0.414$$

Furthermore, we can calculate:

$$z_{os} = z_{ol} \left( \frac{k_{os}}{k_{ol}} \right) \left( \frac{p_{ol}}{p_{os}} \right) = 0.5 \cdot 1.0735 \frac{1}{1.00} = 0.537$$

$$\mu_{os} = 1 - \left( \frac{\gamma_{ol}}{\gamma_b} \right) \left( \frac{\gamma_{os}}{\gamma_{ol}} \right) = 1 - 1.0475 \cdot 1.025 = -0.072$$

From the experiments we find

$$q_{ls} = 1 - \frac{\text{inulin clearance}}{\text{diodrast clearance}} = 1 - \frac{81.4}{320} = 0.736$$

By re-writing we now get from (21)

$$\frac{1 - q_{ls}^2}{B_s} = \frac{1 - y_{ls}^2}{A_s^2} - \frac{2z_{os} - \mu_{os}(1 + y_{ls})}{A_s}$$

This is a 2nd degree equation for the determination of  $y_{ls}$ . If the equation is solved with reference to  $y_{ls}$  we get:

$$y_{ls} = \frac{1}{2} \mu_{os} A_s + \sqrt{\frac{1}{4} \mu_{os}^2 \cdot A_s^2 + \left( 1 - \frac{A_s^2}{B_s} (1 - q_{ls}^2) - A_s (2z_{os} - \mu_{os}) \right)}$$

In the right side of the equation all the values are known, and we get on inserting:

$$y_{ls} = -0.009 + \sqrt{1 - 0.140 \cdot 0.458 - 0.241 (1.074 + 0.072)} = 0.804$$

We have for  $z_{ls}$

$$z_{ls} = \frac{z_{os}}{q_{ls}} = \frac{0.537}{0.736} = 0.730$$

$A_s$  and  $B_s$  being known, we can calculate the coefficients of  $x$  in equation (26); we then find:

$$y = 1 - 0.258x + 0.0959x^2 - 0.0473x^3 + 0.00240x^4 + 0.00312x^5 + 0.00170x^6 + \dots$$

$$q = 1 - 0.742x + 0.548x^2 - 0.0371x^3 - 0.0602x^4 - 0.0387x^5 + \dots$$

The expression for  $q$  is not particularly exact for  $x$ -values in the vicinity of 1, and therefore we must here employ the exact expression (9a) with  $\frac{dy}{dx}$  introduced according to (17). For this case too fig. 4 shows curves for the hydraulic and colloid osmotic pressures. For the erect posture the pressure curves are plotted in stippled lines.

Fig. 4 thus shows the course of the hydraulic and colloid osmotic pressure curves through a single glomerular capillary, it being assumed that for the horizontal posture there is filtration-pressure equiponderance at the capillary terminal, as assumed by SMITH

and co-workers (1940). Fig. 4 also shows the course of the same curves in the passive erect posture; here it will be observed that the colloid osmotic pressure curve is displaced upwards as a consequence of the higher protein concentration. As the mean blood pressure in this experiment did not change, we estimated no change in the initial hydraulic pressure. It should be remarked particularly that at the outlet of the glomerular capillary the hydraulic pressure is now greater than the colloid osmotic pressure, which means that there cannot be pressure equiponderance in the passive erect position. Finally, one notices that the hydraulic pressure at the outlet of the capillary is greater in the passive erect posture than in the horizontal. This increase of pressure can only be explained as being due to the contraction of *vas efferens* in the erect posture. It is impossible to decide whether simultaneously there are changes in the tone of *vas afferens*, as we know nothing of the absolute value of the pressure fall between the aorta and the beginning of the glomerular capillary. Regulation of *vas afferens* is no necessary explanation of the experimental results, but it cannot be precluded.

We shall now turn to a discussion of the values that have not been measured, viz. the hydraulic and colloid osmotic pressures at inflow and outflow. Had we employed other and equally plausible values for these pressures, this would not in principle alter the result of our calculations.

If instead of assuming that there is pressure equiponderance at the terminal of the capillary in the horizontal posture we had assumed that this equiponderance had not yet set in, it is still farther off than ever in the passive erect posture, so that we can still demonstrate a pressure increase at the terminal of the glomerular capillary.

Now had we assumed that pressure equiponderance in the horizontal posture had occurred at a point before the end of the capillary, we should have to assume that the pressure curves ran parallel in their further course. Under this assumption equiponderance in the erect posture will occur at a point nearer the end of the tube than in the horizontal.

As was stated, we have reckoned with an experiment in which the blood pressure did not rise in the erect posture. If it had, we should have been obliged to assume a still greater change in the diameter of *vas efferens* in order to explain the clearance changes observed.

## Conclusion.

The result of the calculations is that the clearance changes observed cannot have been caused solely by general changes in the pressure and composition of the blood, but that a contraction of *vas efferens* must simultaneously be assumed. A regulation of *vas afferens* is not necessary to explain the results of the experiments, but it cannot be precluded. Furthermore the probability is made evident that if pressure equiponderance occurs in the horizontal posture, it does not occur in the erect posture.

## Summary.

1) In some experiments on the renal function in the horizontal and erect postures the authors observed certain changes in the inulin clearance (glomerular filtration) and the diodrast clearance (renal blood flow) which, compared with the changes occurring in the erect posture in the pressure and composition of the blood (colloid osmotic pressure, cell volume and viscosity), necessitated penetrating into the problems of glomerular hydrodynamics.

2) The hydraulic problem is the following: What laws govern the laminar flow of colloidal solutions in semi-permeable tubes?

3) Formulae are deduced for flow, filtration, hydraulic and colloid osmotic pressure in a semi-permeable tube perfused by a colloidal solution.

4) The validity of the formulae for water and plasma is proved by model experiments.

5) The formulae are employed for calculating the results of a concrete experiment made to determine the renal function in the passive erect posture. From these calculations the authors plot the course of the hydraulic and colloid osmotic pressures in a glomerular capillary for horizontal and erect postures.

The course of these curves shows that a contraction of *vas efferens* must take place in order that the observed simultaneous changes in the inulin and diodrast clearances and in the composition and pressure of the blood may occur.

The authors wish to express to Professor, Dr. phil. l. BRANDT-REHBERG their gratitude for inspiring discussions and valuable assistance.



## Explanation of symbols.

- $a$  = the protein volume entering per second at the inflow of the tube.  
 $a_n$  = auxiliary value for the calculation of  $\beta$ . (Formula 25).  
 $A$  = fundamental calculation constant. (Formula 10).  
 $b$  = width of channel cross-section in model.  
 $b_n$  = auxiliary value for calculating  $\beta$ . Formula (25)  
 $B$  = fundamental calculation constant. Formula (14).  
 $C$  = coefficient of permeability of tube wall.  
 $C_n$  = auxiliary value for calculation of  $\beta$ . Formula (25).  
 $f\%$  = filtration fraction.  
 $F$  = the absolute filtration quantity for whole tube length.  
 $FC$  = Fluid Column.  
 $g$  = acceleration of gravity.  
 $h$  = height of channel cross-section in model.  
 $I$  = Fall on the hydraulic pressure line.  
 $k$  = the colloid osmotic pressure at a given point of the tube.  
 $k_0$  = the colloid osmotic pressure at the inflow.  
 $l$  = abscissa for any point of the tube.  
 $L$  = length of tube.  
 $m$  = factor of proportionality between protein concentration and colloid osmotic pressure.  
 $p$  = the hydraulic pressure at a given point of the tube.  
 $p_0$  = the hydraulic pressure at the inflow.  
 $p_c$  = capsular pressure.  
 $q$  = fluid flow at a given point of the tube in relation to the flow at the inflow.  
 $q_1$  = fluid flow at the outflow of the tube in relation to that at the inflow.  
 $Q$  = fluid flow at a given point.  
 $Q_0$  = fluid flow at the inflow.  
 $r$  = radius of tube.  
 $x$  = abscissa for a given point of the tube in relation to the entire length of the tube.  
 $y$  = the hydraulic pressure at a given point of the tube in relation to the pressure at the inflow.  
 $y_1$  = the hydraulic pressure at the outflow of the tube in relation to the pressure at the inflow.  
 $z$  = the colloid osmotic pressure at a given point of the tube in relation to the hydraulic pressure at the inflow.  
 $z_0$  = the colloid osmotic pressure at the inflow in relation to the hydraulic pressure at the inflow.  
 $\alpha$  = the protein concentration at a given point (by volume).  
 $\alpha_0$  = the protein concentration at the inflow (by volume).  
 $\beta$  = coefficients of  $x$  in a series determining  $y$ -formula (22).  
 $\gamma$  = the sp. gravity of the solution at a given point of the tube.  
 $\gamma_0$  = the sp. gravity of the solution at the inflow.  
 $\gamma_a$  = the sp. gravity of the protein.  
 $\gamma_b$  = the sp. gravity of the water phase.

$\eta$  = the absolute viscosity.

$\mu$  = Calculation value. Formula (6a).

$\nu$  = the kinematic viscosity of the solution.  $\frac{g \times \eta}{\gamma} g$  = gravity acceleration,  $\eta$  = the relative viscosity.

$\nu_{20}$  = the kinematic viscosity of the water at 20° C.

$\theta$  = the calculation value having an influence on the calculation of the water tests. Formula (35).

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## On the Influence of the Neurohypophyseal Principles on the Sodium Metabolism in the Axolotl (*Amblystoma mexicanum*).

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### Introduction.

The ability of many fresh water animals to take up certain ions, especially  $\text{Cl}^-$  and  $\text{Na}^+$ , from the surrounding medium has been well established, mainly by the work of KROGH and his collaborators (KROGH 1939).

The uptake may take place through the gills (fishes), through special organs (mosquito larvae) or through the whole surface (frog); in species where this mechanism of salt uptake is well developed (frog, goldfish) ions may be taken up even from 0.01 millimolar solution, the salt thus being concentrated about 10,000 times on the passage from the medium into the blood of the animal.

This mechanism is regulated: KROGH (1937 a) found that frogs, which had lost salt during a several days' stay in repeatedly renewed water, take up NaCl eagerly when placed in dilute NaCl-solutions, whereas frogs which had not lost salt do not show any net uptake of salt under similar conditions.

Other forms investigated, whether insect larvae (KOCH 1938) or fishes, behave in the same manner (KROGH 1937 b, 1938). There can thus be no doubt that the rate of uptake is regulated according to the requirements. However, the regulation mechanism has hitherto attracted but little attention.

At the start of the present study of the regulation of the salt metabolism, three possibilities out of many were considered, viz.

1) Chemoreceptors in the nervous system are influenced by the concentration of the ions in question and regulate the activity of the ion transporting cells through nervous action.

2) Under the influence of the ions the supposed chemoreceptors induce the formation of a special hormone or hormones which, in turn, sets the pace of the ion transporting cells.

3) Changes in the concentration of the ions in question act directly upon the ion transporting cells so as to induce appropriate changes in the uptake.

Since for different groups of animals adaptation to limnic life has evidently taken place independently, the regulation mechanism need not necessarily be identical in all forms. The present study was performed on one species only, namely the axolotl. It was decided to begin the investigation with the second of the above mentioned assumptions, the hormonal regulation, because general considerations show that animals frequently use hormones rather than direct nervous action when a stimulus is to be applied during longer periods.

As a matter of fact, the first hormone preparation tested — a posterior lobe extract from ox — had a pronounced effect on the salt uptake in the axolotl; the present paper deals with this influence of the posterior lobe principles on the uptake of sodium and chlorine in the axolotl.

### Methods.

During the experiments each animal was kept in a glass chamber of the type shown in Fig. 1 containing 200—300 ml 1/50—1/150 frog's Ringer solution.

The liquid is kept circulating by a constant stream of moist air entering through the tube A and escaping through the aperture B, thus also renewing the air in the space above the liquid. (This is of importance because the animals have an auxiliary mouth cavity respiration.)

The samples for analysis are drawn through B.

Radioactive sodium chloride was added to trace the absolute movements of ions into and from the animal.

The experimental animals were fully grown (unmetarmorphosed) 3—4 years old specimens of axolotl (*Amblystoma mexicanum*) weighing between 65 and 103 g. When not in experiment, they were kept in stone ware jars with tap water and were fed with calf's liver.

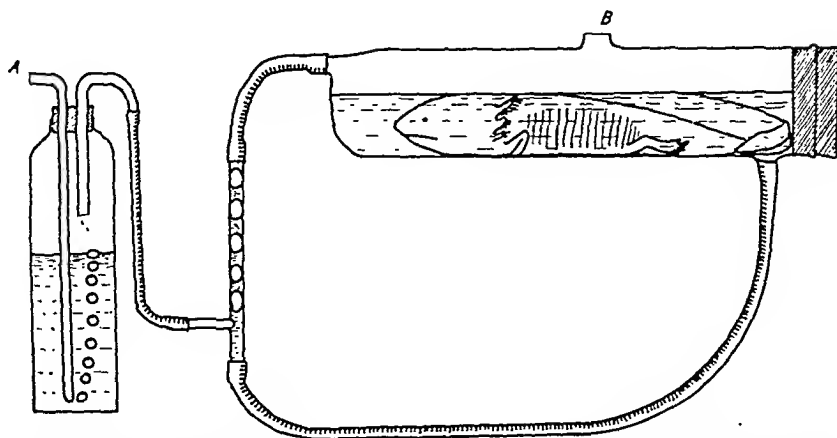


Fig. 1. Experimental chamber containing an axolotl. The liquid is kept circulating by means of an air stream entering the washing bottle through the inlet A. Samples drawn through the aperture B.

About a fortnight prior to the experiments, the feeding was suspended in order to allow the animals to empty their guts; without this precaution the tubes of the circulation system might be plugged with feces.

*Chlorine* was determined according to SCHNOHR (1934) on 1 ml samples.

For *sodium* determinations the method of HOFFMAN and OSGOD (1938) was used in a slightly modified form. The aqueous samples — usually 8 ml each — were evaporated to dryness on a steam bath in centrifuge tubes, using a special hot air evaporation apparatus (see SCHMIDT-NIELSEN 1945) to speed up the drying process. Ashing was omitted in view of the very low content of organic matter in the circulating medium. 8 ml zinc uranyl acetate reagent were added to each tube and thorough mixing was secured by using a mechanical stirring set handling twelve analyses simultaneously. From this point, the analyses were performed as outlined by HOFFMAN and OSGOD (l. c.), except for the use of the mechanical stirrer after every addition of washing fluids.

Since the sodium concentrations often were considerably lower than those for which the original method was worked out, a calibration curve was applied where the concentration was plotted against photometer readings between 0 and 0.4  $\mu$ equiv. of sodium. The calibration curve is shown in Fig. 2.

Geiger-Müller counters were used for the radioactive measurements. The samples — usually 0.5 ml of the solution — were transferred to aluminium dishes by means of a Krogh syringe and were evaporated to dryness in an electric oven at 105 ° C. The dishes were greased with vaseline on the edges in order to prevent creeping of the solution during the drying process.

In a few experiments with radio-chlorine, 20  $\mu$ mol of inactive NaCl were added to each sample and the Cl was precipitated with an excess

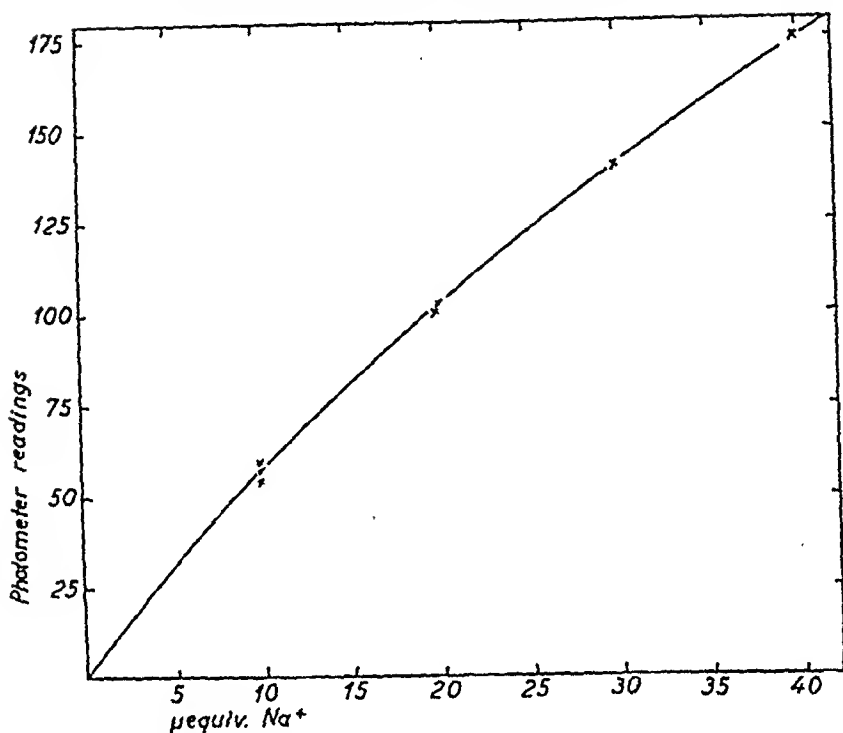


Fig. 2. Calibration curve for the colorimetric sodium determination.

of  $\text{AgNO}_3$ . After washing, the precipitate was transferred to counting dishes.

Radioactive Na and Cl were prepared in the Copenhagen cyclotron by deuteron bombardment of NaCl. The  $\text{Na}^*$  countings were postponed until the very short living  $\text{Cl}^*$  had faded away.

As the preparations generally contain traces of  $\text{Cu}^*$  from the copper target (the plate carrying the NaCl during bombardment), the solution of the irradiated NaCl was treated with charcoal in order to remove traces of heavy metals.

The decay of the labelled Na was followed in most cases and was found to agree with the half-life period of sodium stated in the literature.

### Calculation of the Passage of Ions.

The experiments were performed with very dilute solutions, viz. 1–3 millinormal with respect to sodium and chlorine. On the other hand, the concentration of these ions in the blood of the animals is about 100 millinormal, so that diffusion out — in the classical sense of the word — will proceed 30–100 times faster than diffusion in. Relative to the rate of active uptake, diffusion may therefore be neglected as a cause of disappearance of radioactive ions from the solution. The

equations for the rate of active uptake derived in the following are based on the assumption that the ions leaving the animal (by diffusion or with the urine) do not carry with them any significant amount of radioactive ions. This assumption is permissible if the outer activity of the medium is not allowed to decrease too much during the experimental period. The total amount of sodium ions in the animals being much higher than the total amount in the surrounding medium, the active ions taken up are diluted with the large amount of inactive ions inside the animal.

On the assumptions mentioned previously, the active uptake of a given ion may be calculated as follows. The relative radioactivity per unit volume may be denoted as  $y$  and the total amount of the ion in question as  $A$ .

The rate of active uptake, *i. e.* the amount of the ion in question which passes into the animal per unit time is  $\frac{di}{dt}$ , whereas the rate of decrease of the activity is  $\frac{dy}{dt}$ .

Since the ion absorbing cells cannot distinguish between active and inactive ions, we have

$$-\frac{\frac{dy}{dt}}{\frac{di}{dt}} = \frac{y}{A} \dots \dots \dots (1)$$

or

$$-\frac{dy}{dt} = \frac{di}{dt} \cdot \frac{y}{A} \dots \dots \dots (2)$$

$\frac{di}{dt}$  is not necessarily equal to the rate of change in  $A$ ,  $\frac{dA}{dt}$ , because the animal may excrete the ion in question so that  $A$  even may increase with time. An infinitesimal change in  $A$  in whatever direction during an infinitesimal time is, however, immaterial for the validity of (2).

If  $\frac{di}{dt}$ , the rate of active uptake, is taken as a constant during the experimental period, we may replace  $\frac{di}{dt}$  by the constant  $U$  and thus obtain

$$\frac{dy}{dt} = -\frac{U}{A} \cdot y \dots \dots \dots (3)$$

For our purpose, it may suffice to consider two cases, viz. (I)  $A = \text{constant}$  and (II)  $A$  is increasing or decreasing at a constant rate.

For  $A = \text{constant}$ , we get

$$y = y_0 e^{-\frac{U}{A}t} \text{ or } U = -\frac{A}{t \log e} \log \frac{y}{y_0} \dots \dots \dots (4)$$

Insertion of the known values of  $y$ ,  $y_0$ ,  $A$ , and  $t$  gives the rate of active uptake  $U$ .

If a net uptake or excretion of the ion has occurred, we must subdivide the experiment in periods during which this net change proceeds at an approximately constant rate,  $B$ , where  $B$  is positive when the quantity of the ion in the medium increases.

We have

$$A = A_0 + Bt \quad \dots \dots \dots (5)$$

Inserting (5) in (3) we get

$$\frac{dy}{dt} = -y \frac{U}{A_0 + Bt} \quad \dots \dots \dots (6)$$

Solving this differential equation we get

$$y = e^{\frac{U}{B} \ln(A_0 - Bt) + K} \quad \dots \dots \dots (7)$$

where  $K$  is an integration constant, or

$$y = e^K (A_0 - Bt)^{U/B} \quad \dots \dots \dots (8)$$

inserting the values for the time  $t$  and the starting moment (designated  $y_0$  and  $A_0$ ), we get

$$\frac{y_t}{y_0} = \frac{e^K (A_0 - Bt)^{U/B}}{e^K A_0^{U/B}} = \frac{A_t^{U/B}}{A_0^{U/B}} \quad \dots \dots \dots (9)$$

From (9) we get (10)

$$U = B \frac{\log \frac{y_0}{y_t}}{\log \frac{A_0}{A_t}} \quad \dots \dots \dots (10)$$

directly leading to the rate of active uptake,  $U$ .

As mentioned above, these equations no longer hold if significant amounts of radioactive ions are retransferred from the animal to the solution. Therefore, the equations have only been applied without correction if the activity of the outer solution had decreased by less than 50 % of the starting activity.

In cases where the rate of active uptake had to be followed over a longer period, the principle of the experimental technique was to increase the activity of the outer solution whenever the ratio-Na content within the animal approached relatively significant values. After every change of the solution to one of say 5—10 times higher activity (but nearly the same Na-concentration), the inner activity again becomes insignificant.

In the tables, the rate of active uptake is expressed in microequivalents taken up per 24 hours even in cases where the experimental period has been shorter than 24 hours. The true excretion,  $E$ , including both the amount diffused out and that excreted with the urine, is determined as  $E = U + B$ .



## Results.

## 1) Net Changes in the Sodium and Chlorine Content of Normal Animals.

In many cases, normal animals have been kept in the chambers for weeks in order to follow spontaneous changes in the Cl- and Na-content of the animals. In the media used (containing 1–3 millimolar NaCl) the salt content of the animals may remain practically constant for days and even for weeks (cf. Fig. 3). Often,

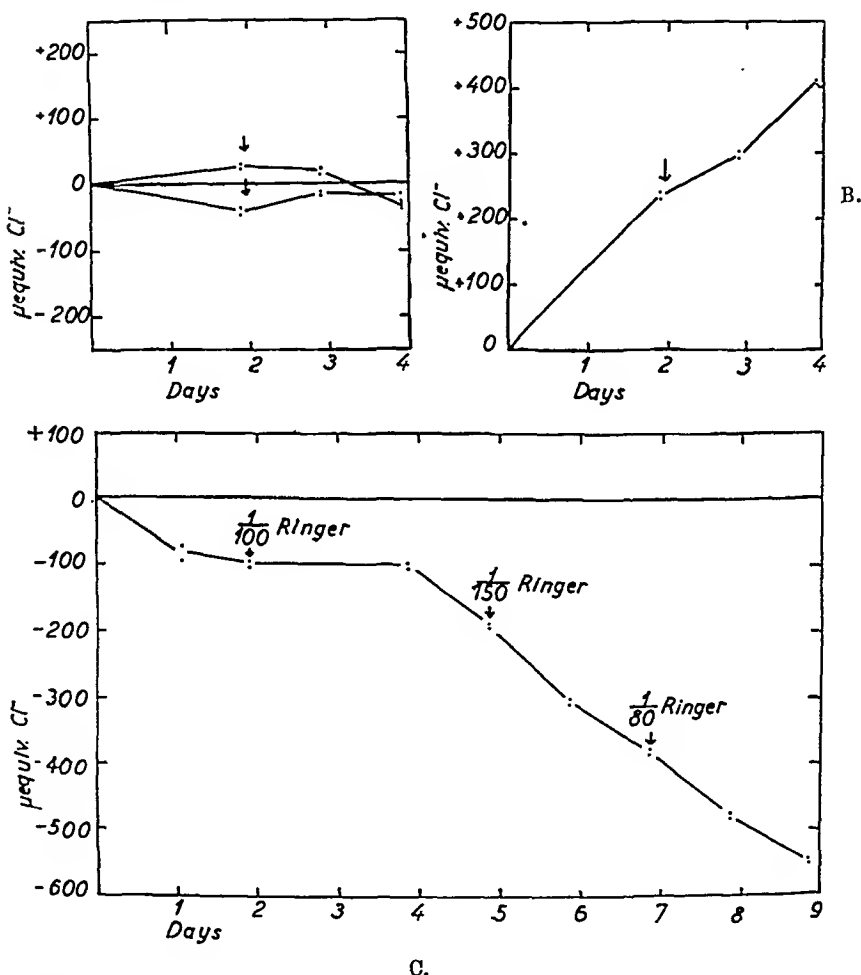


Fig. 3 (A, B, C). Spontaneous uptake and excretion of chlorine. The axolotls are kept in diluted frog's Ringer. The arrows indicate renewal of the medium.

however, spontaneous changes, *i. e.* both a net uptake and a net loss of salt were observed, which may last for several days (Fig. 3 B, C). The highest rate of spontaneous net uptake of salt noticed is  $150 \mu\text{mol}/24$  hours for sodium and  $154 \mu\text{mol}/24$  hours for chlorine. However, even values of about  $100 \mu\text{mol}/24$  hours are met with only in few cases. The highest rate of net salt loss observed was  $181 \mu\text{mol}/24$  hours (Cl) and  $106 \mu\text{mol}/24$  hours (Na). In the case of the highest Cl-loss the Na-loss has not been determined.

For our hormone-experiments some animals have been excluded, *viz.* those which had shown a pronounced tendency to spontaneous reactions during a preceding period.

In most experiments to be described in the following, normal periods lasting for one or several days are presented in the figures. These comparison periods give good evidence that the spontaneous fluctuations in the Na- and Cl-content as a rule are small compared with the hormonally induced changes.

## *2) The Absolute Values for Sodium Uptake and Excretion in Normal Animals.*

Using radio-sodium as an indicator, the uptake and the excretion of sodium have been determined on 17 normal animals, 7 females and 10 males. Altogether 61 determinations have been made. Fig. 4 shows the distribution of the determinations divided into classes differing by  $25 \mu\text{equiv.}/24$  hours. It appears that more than 80 % of the determinations fall between  $25-100 \mu\text{equiv.}/\text{day}$ . The mean value is  $68.7 \pm 4.2 \mu\text{equiv.}/\text{day}$ . The corresponding distribution of the excretion determinations is shown in Fig. 5.

The mean of the daily excretions is  $94.6 \pm 5.3 \mu\text{equiv.}/\text{day}$ . These values might indicate a tendency of the animals to lose more salt than is taken up while they are kept in the chambers. The loss may perhaps be due to the fact that the animals starve and, therefore, lose in weight on an average 0.45 g or about 0.5 % per day. The net loss of sodium is approximately  $20 \mu\text{equiv.}/\text{day}$ . If the total content of sodium is taken to be  $2000-5000 \mu\text{equiv.}$ , *cf.* p. 366 the salt loss corresponds very well to the loss in weight.

In one case, a group of four animals gave quite aberrant values for the uptake and excretion, the uptakes varying from  $120-290 \mu\text{equiv.}$  per day, and the excretions being correspondingly high. It is, however, doubtful whether these animals should be regarded as normal, because by an accident they were submitted to a

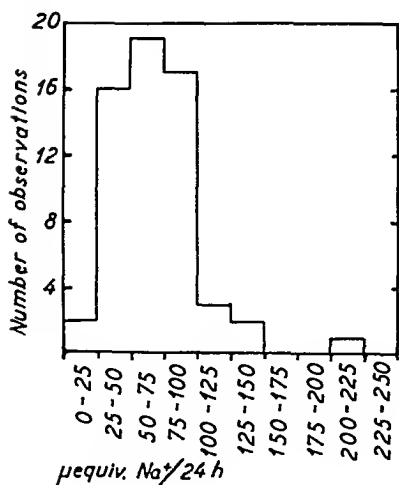


Fig. 4. Normal sodium uptake per 24 h in axolotls weighing from 65 to 103 g.

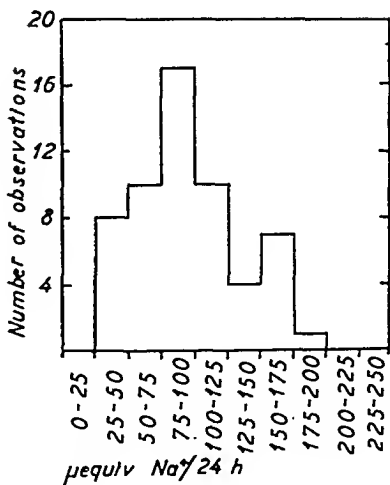


Fig. 5. Normal excretion of sodium per 24 h in axolotls weighing from 65 to 103 g.

period of insufficient oxygen supply just at the beginning of the experiment. On other occasions we have seen that want of oxygen induces a rapid loss of salt which, when oxygen is again supplied, is followed by a rapid uptake. The group in question was therefore rejected in the calculation of the mean uptake and excretion.

### 3) *The Influence of Unfractionated Neurohypophyseal Extracts on the Salt Equilibrium.*

A female axolotl, weighing 96 g, was kept in 1/80 Ringer solution for four days without showing any net uptake or excretion of chlorine. Subsequently, 0.25 ml Pituitin (corresponding to 2.5 international units of neurohypophyseal hormones) was injected subcutaneously and the animal was placed in 300 ml 1/80 Ringer.

It appears from Fig. 6 that this injection — after a transient small loss — resulted in a pronounced uptake of  $\text{Cl}^-$  which lasted for more than two days. The  $\text{Cl}^-$  concentration of the solution decreased from 1.60 to 0.70 mequiv. Then, the excretion began to exceed the uptake, and this continued until the animal had lost more  $\text{Cl}^-$  than it had taken up after the injection.

The experiment was repeated with practically the same result. Thus, neurohypophyseal extracts obviously influence the  $\text{Cl}^-$  content of the axolotl. However, it remained to be settled whether

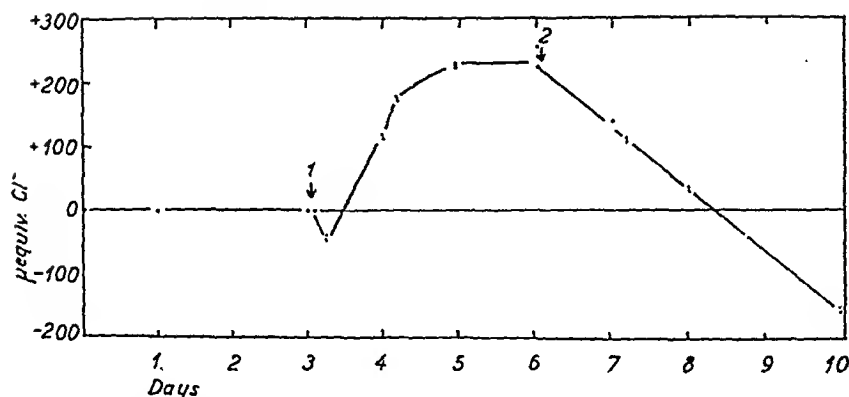


Fig. 6. Influence of Pituitin (unfractionated extract of the neurohypophysis) on the balance of chlorine in the axolotl. Net uptake and excretion in  $\mu\text{equiv.}$  Diluted frog's Ringer renewed at the arrows. At arrow 1, injection of 0.25 ml Pituitin (2.5 int. units).

this net uptake was due to an increased uptake or to a reduced loss. Moreover, it had to be found out which of the physiologically active principles or hormones of the neurohypophysis was responsible for the action observed.

#### 4) Influence of the Pressor Fraction.

Injection of 0.25 ml of a pressor preparation (Insipidin), corresponding to 5 pressor units, called forth reactions which were quite similar to those produced by Pituitin. Fig. 7 is typical of the Insipidin reaction. The changes of the  $\text{Na}^+$  and  $\text{Cl}^-$  were followed. Both ions are given off to a small extent in the course of the first 6 hours after injection; subsequently, a more or less rapid uptake sets in, which lasts for three to six days and is followed — just as it was the case with Pituitin — by a loss of salt lasting for several days. The more protracted course of the Insipidin reaction may be due to the lower temperature,  $15^\circ\text{--}17^\circ\text{C}$ , compared with  $23^\circ\text{--}24^\circ\text{C}$  in the case of the Pituitin experiments.

In the first part of the curves (Figs. 6, 7, 8) gain and loss of  $\text{Na}^+$  and  $\text{Cl}^-$  are plotted, using the content of these ions in the animals at the start of the comparison period as a basis; in the second part of the curves the basis is the content at the moment of injection.

The moments when the solutions were changed are indicated with arrows. In a few cases, doses smaller than 0.25 ml Insipidin have been injected. 0.05 ml gave a just perceptible reaction.

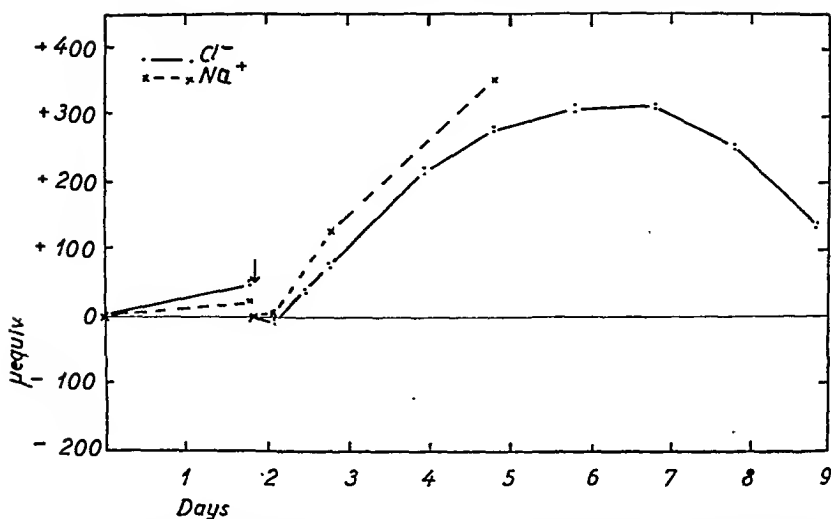


Fig. 7. Influence of Insipidin (pressor principle of the neurohypophysis) on the balance of chlorine and sodium in the axolotl. Net uptake and excretion in  $\mu$ equiv. At the arrow, injection of 0.25 ml Insipidin (5 int. units) and renewal of diluted frog's Ringer.

##### 5) Influence of the Oxytocic Fraction.

On all animals tested, injection of the oxytocic fraction produced a rapid net loss of sodium and chlorine. An example for the effect of the injection of 0.25 ml Pitupartin (corresponding to 2.5 international units of oxytocic principle) on axolotls is given in Fig. 8. Sooner or later, the loss is followed by a net uptake, in some cases restoring the initial salt content of the animals. In two cases (out of c. 20), the animals did not recover after the injection of Pitupartin. They continued to lose salt (interrupted in one case by a short period of intense uptake) and, finally, they died, possibly as a consequence of the salt loss.

##### 6) The True Uptake and Excretion of Sodium after Insipidin Injection.

The experiments described above show that a net uptake of salt is induced by the pressor fraction, whereas the oxytocic fraction induces a net salt loss in the axolotl.

In order to decide to what extent these changes of the salt content were due to changes of the active uptake and, moreover, to what extent alterations of the salt excretion were responsible for the observed phenomena, the true movements of the sodium ions were followed by means of radio-sodium.

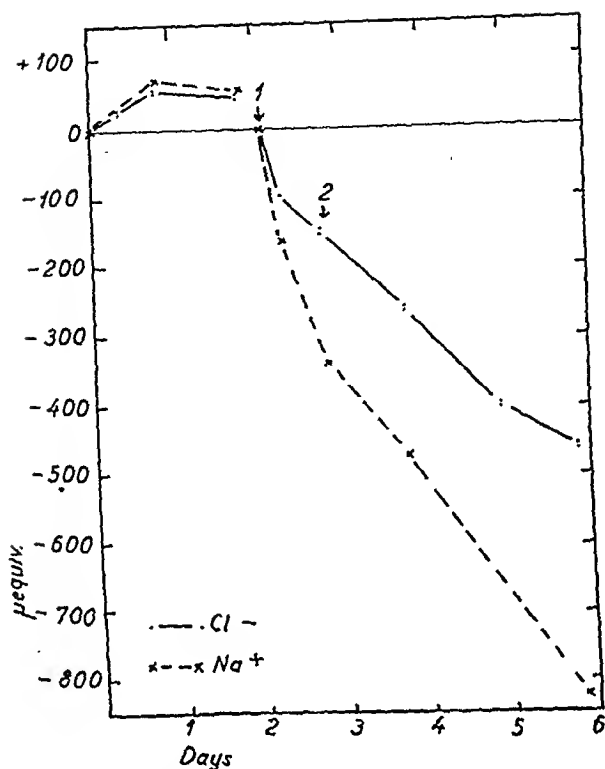


Fig. 8. Influence of Pitupartin (oxytocic principle of the neurohypophysis) on the balance of chlorine and sodium in the axolotl. Net uptake and excretion in  $\mu\text{equiv.}$  Diluted frog's Ringer renewed at the arrows. At the arrow, injection of 0.25 ml Pitupartin (2.5 int. units) and renewal of diluted frog's Ringer.

In Table I the uptake and excretion of sodium during the last 24 hours prior to an Insipidin injection are compared with the corresponding values for the first 24 hours after injection. The uptake increased by 200 %. The excretion, however, increased by about 100 %, the net gain thus being about 100 % of the normal daily uptake.

As mentioned above, Insipidin injection as a rule produces net loss of sodium and chlorine during the first 6 hours' period. This loss is due to an increased excretion (in any case with respect to sodium) whereas the active uptake is already increased, although not sufficiently to balance the rapid loss (see Table II).

Table II also shows that the active uptake is still very high in the second 24 hours' period after injection, in which the excretion has decreased to normal. The rate of net gain is, therefore, at its maximum about 24 hours after the injection.

Table I.

*Effect of Insipidin on uptake and excretion of sodium.*

Experiment No.	$\mu$ equiv. Na taken up per 24 hours		% increase in Na-uptake on Insipidin injection	$\mu$ equiv. Na excreted per 24 hours		% increase in Na-excretion on Insipidin injection
	before Insipidin injection	after Insipidin injection		before Insipidin injection	after Insipidin injection	
I ...	99	275	178	153	251	64
II ...	55	191	247	107	95	—11
III ...	122	324	156	41	162	295
IV ...	79	205	160	57	78	37
V ...	66	174	164	47	133	183
VI ...	50	246	390	80	245	206
VII ...	61	173	173	98	173	77
Mean	76	227	200	83	162	95

Table II.

*Effect of Insipidin on the uptake of sodium.*

Experiment No.	$\mu$ equiv. Na taken up per 24 hours		
	before Insipidin injection	first 6 h period after Insipidin injection	second 24 h period after Insipidin injection
II.....	55	200	292
III.....	122	133	242
IV.....	79	160	250
V.....	66	63	116
VI.....	50	192	346
VII.....	61	160	186
Mean	72	151	239

### 7) The True Uptake and Excretion of Sodium after Pitupartin Injection.

Just as it was the case with Insipidin, Pitupartin during the first 6 hours after injection produces an increase in the uptake as well as in the excretion of sodium. This is clearly seen from Figs. 9 and 10. Whereas in the case of Insipidin the augmentation of the excretion vanishes very soon, the opposite is the case after Pitupartin injection. The uptake decreases to normal values or even lower, whereas the excretion remains very high for several days.

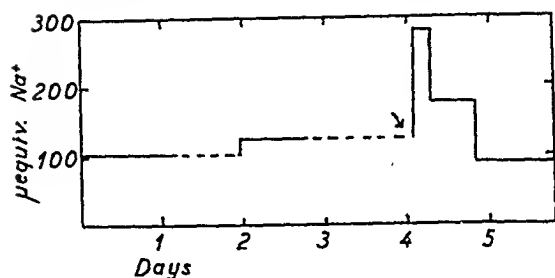


Fig. 9. Influence of Pitupartin on the true uptake of sodium in the axolotl. At the arrow, 0.25 ml Pitupartin was injected. (Mean of three animals.)

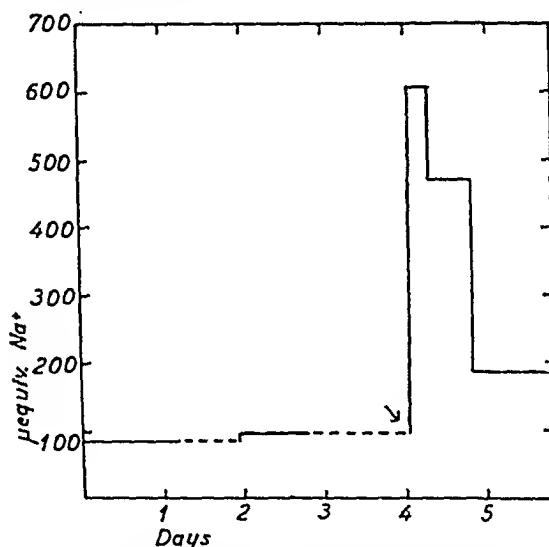


Fig. 10. Influence of Pitupartin on the true excretion of sodium from the axolotl. At the arrow, 0.25 ml Pitupartin was injected. (Mean of three animals.)

The animals show considerable deviations as to the span of time required for recovery after a Pitupartin injection; but the essential point in the reaction is evidently an increased excretion rather than a decrease in the active uptake.

#### 8) *The Influence of Salt Injections on the Sodium Turnover. The "Pricking Effect".*

Whereas KROHG's experiments have shown that a reduction of the salt content of fresh water animals induces an increased uptake of salt, no experimental evidence has hitherto been collected as to the question whether an increased salt content will depress



the active uptake. Thus, when we got a group of animals showing an unusually high spontaneous sodium turnover (cf. above p. 8), it was decided to increase their salt content by injection of a hypertonic salt solution in order to find out whether this would depress the active uptake of sodium.

The immediate result of the salt injection (0.5 ml 1.8 % NaCl) was in all cases a sudden increase lasting for about 6 hours. Then, the values for the uptake became similar to those before the injection, but within the span of time in which the active uptake

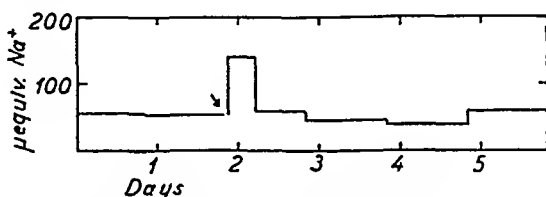


Fig. 11. Influence of injections of hypertonic NaCl-solution on the true uptake of sodium in the axolotl. At the arrow, 4 % NaCl was injected. (Mean of four animals.)

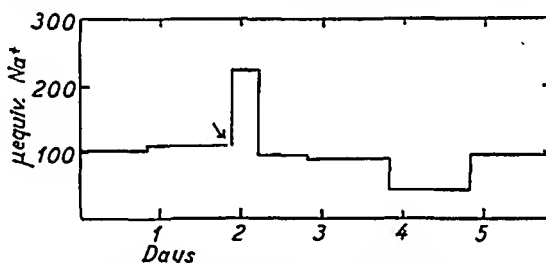


Fig. 12. Influence of injections of hypertonic NaCl-solution on the true excretion of sodium in the axolotl. At the arrow, 4 % NaCl was injected. (Mean of four animals.)

was followed with radio-sodium, there was but little indication of a depression of the active uptake below the initial values. The excretion showed a similar transient increase immediately after the injection.

The experiment was repeated with four normal animals. Two of them received 1 ml 4 % NaCl and two 0.5 ml 4 % NaCl. Fig. 11 reveals that the most pronounced effect of the salt injection is the brief increase in sodium uptake.

The influence of salt injections on the sodium excretion is shown in Fig. 12; here, too, the injection of NaCl was immediately followed by an increase of a few hours' duration. In one of the animals receiving 1 ml 4 % NaCl the initial increase was lacking. This,

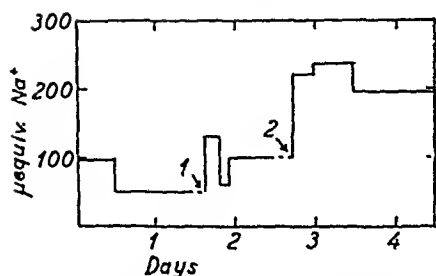


Fig. 13. Influence of pricking of the skin on the true uptake of sodium in the axolotl. At arrow 1, the animals were pricked with an injection needle; at arrow 2, 0.25 ml Insipidin was injected (cf. the text below). (Mean of four animals.)

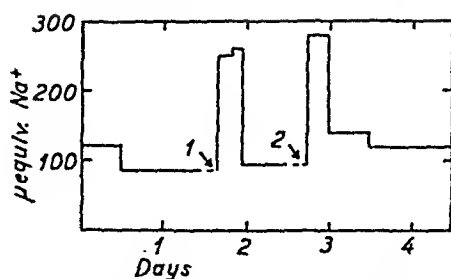


Fig. 14. Influence of pricking of the skin on the true excretion of sodium from the axolotl. At arrow 1, the animals were pricked with an injection needle; at arrow 2, 0.25 ml Insipidin was injected. (Mean of four animals.)

however, is the only case out of 12 where salt injection did not bring about a shortlasting rise of salt excretion.

In this connection, it should be emphasized that the excretion values are calculated as the sum of the uptake and the absolute increase in outer concentration and, thus, are encumbered with a greater uncertainty than the values for the uptake.

The net result of the simultaneous changes in uptake and excretion is insignificant. The animals may even continue to gain sodium and chlorine after the injections.

These experiments seem to disprove the assumption that the concentration of sodium or chlorine ions in the body fluids determines the rate of active uptake through a direct action of the ions on the transporting cells. Moreover, the experiments suggest that the injection itself, independent of the substance injected, may induce a brief, but intense increase in uptake as well as in excretion of sodium (and, probably, chlorine as well).

The next experiment was made in order to check whether the mere introduction of the injection needle suffices to induce the supposed pricking effect (transient increase in uptake as well as in excretion), and, moreover, to compare this effect with that of Insipidin. (See Figs. 13 and 14.)

The pricking effect was found to occur in all four cases both with regard to the uptake and to the excretion, although the reaction was not equally marked in all animals. The difference between the reaction to pricking and to Insipidin injection is very conspicuous, especially when the uptake is considered. The Insipidin reaction is stronger and much more protracted than the pricking effect.

9) *Estimation of the Sodium Content of the Experimental Animals by Isotope Dilution.*

Radio-sodium may be used to determine the sodium content of the animals without killing them. The following experiment may illustrate the procedure applied.

A male axolotl, weighing 96 g, received a 0.25 ml Inspidin injection to speed up the active uptake of sodium. The animal was placed in 250 ml 1/80 Ringer containing radio-sodium of known activity. The changes in activity and in Cl-concentration of the solution were followed by analysing small samples taken daily over a longer period. The animal took up the larger part of the chlorine (see Table III) and the activity decreased to 4.5 % of the initial value. On the fourth day of the experiment, the animal again showed a net loss of salt and on the fifth day the outer concentration of  $\text{Cl}^-$  and  $\text{Na}^+$  had increased above the starting level. This means that from that time practically all sodium present in the solution originated from the animal and, thus, was bound to contain radio-sodium in the same proportion as the sodium inside the animal. Before the animal was placed in the chamber the activity per  $\mu\text{equiv. Na}$  in the solution was 65.6; after the lapse of 5 days the corresponding figure was 4.34, when a small correction is made for the decrease in relative activity due to the removal of the samples. In other words, the sodium originally present in the solution has been diluted with 14 times its quantity of sodium from the animal. The sodium content of the animal was, therefore,  $340 \times 14 = 4760 \mu\text{equiv.}$  at the beginning of the experiment.

Table III.\*

Hours after addition of $\text{Na}^*$	Relative $\text{Na}^*$ activity per ml	$\text{Na}$ conc. milliequiv.	$\text{Cl}$ conc. milliequiv.	Volume
0 .....	100	1.50	1.54	227
18 .....	49.9	—	1.48	227
42 .....	10.8	—	0.55	224
65.5 .....	4.5	—	0.37	221
90 .....	7.8	1.85	1.15	218
113 .....	13.9	3.21	2.48	195

\* For explanation cf. the text above.

## Discussion.

The experiments presented in the experimental part show a pronounced effect of the neurohypophyseal principles on the salt metabolism of the axolotl.

The pressor principle (represented by Insipidin) induces a net uptake of sodium and chlorine, whereas the oxytocic principle (represented by Pitupartin) induces a rapid salt loss.

The application of radio-sodium revealed that both net uptake and net loss are rather complex processes, being the resultants of changes in true uptake as well as in excretion.

Considering in the first place the Insipidin reaction, we have an increase in excretion as well as in uptake during the first 6 hours after injection. This effect on the excretion first seemed a serious obstacle, if the pressor principle was to be regarded as having a physiological function in the regulation of active salt uptake. There is, however, good reason to believe that the transient increase in the excretion is not due to the pressor principle itself.

As mentioned above, the injection of a salt solution or even the mere pricking of the skin of the animals induced a quite similar transient increase in both the excretion and the uptake of sodium.

Moreover, it is well known that even the best pressor principle preparations are not free from oxytocic principle (cf. KAMM et. al. 1928) and the strong action of oxytocin on the excretion might well be perceptible even in doses, say 10 times smaller than those used to investigate the oxytocin action.

Similarly, we may probably regard the increase in excretion<sup>1</sup> as the main result of the injection of oxytocic principle, whereas the transient initial increase in uptake might be a pricking effect, perhaps combined with the action of a minor amount of pressor principle present as impurity in the Pitupartin used.

In this connection it is of interest that oxytocin seems to induce an increased excretion of chlorine in the mammalian kidney (cf. DICKER and HELLER 1946). It is not unlikely that in the axolotl, too, oxytocin acts upon the kidney tubules so as to reduce the reabsorption of chlorine and sodium. Experiments are planned, however, to distinguish between renal excretion and salt loss through the skin.

It is doubtful whether oxytocin itself has any effect on the

<sup>1</sup> In the present paper, the word excretion has been used in a broad sense, including renal excretion, excretion through skin glands, and loss by diffusion.

active uptake; as soon as more material is available it is our intention to resume this problem on account of its significance for the question whether the active uptake through the skin is of the same nature as the active reabsorption of salt in the kidney tubules.

As mentioned above, salt injection has little effect on the active uptake of sodium, apart from the pricking effect. Although the experiments are too few to give safe evidence, they are very much against the view that the concentration of salt in the body fluids directly determines the activity of the salt transporting cells.

The pricking effect, however, shows the importance of a nervous factor. The increase in active uptake found after salt injection cannot be an adequate reaction to an increase in the salt concentration of the body fluids. On the other hand, it resembles to some extent the release of antidiuretic hormone in dogs after injuries to the skin (RYDIN and VERNEY 1938, O'CONNOR and VERNEY 1942) or in rabbits after irritation of the lumbal vertebrae (HATERIUS 1939). HATERIUS found that this release of the anti-diuretic factor disappeared when the connection between hypothalamus and pituitary was cut.

If it is assumed that the pricking effect is due to a simultaneous release of both neurohypophyseal factors, it would be intelligible that the skin injury induces an increase in uptake as well as in excretion of sodium. Further experiments are needed to elucidate this problem.

In the foregoing, we have spoken of neurohypophyseal principles and not of hormones, because it is still an unsettled problem whether the neurohypophysis produces one or two hormones. Although much evidence is in favour of the view that the pressor and the oxytocic principle are formed as such in the gland (cf. IRVING et. al. 1941) several investigators still hold that only one hormone is formed, the pressor and oxytocic principles being artefacts.

It will be difficult, however, to evaluate the importance of the neurohypophysis for the normal osmoregulation in the axolotl before it is known whether the principles injected in the described experiments are true hormones. The problem may be still more complicated because, as was first observed by BRUNN (1921), neurohypophyseal extracts contain a factor which increases the permeability of amphibian skin to water. This factor is most abundantly present in the oxytocic fraction, but is probably not identical with oxytocin (HELLER 1941).

The Brunn effect is pronounced in toads and frogs, but insignificant in Urodela (cf. HELLER 1945). In the axolotl, we have observed increases in weight of about 4—5 % on Pitupartin injection. The Brunn effect thus is of no importance for our results; but the presence of the Brunn factor reminds of the possibility that more than the known factors may be found in neurohypophyseal extracts.

The rate of active uptake of radio-chlorine has been followed only in a few cases and, due to the short half-life of this isotope, the results are not as exact as those obtained with radio-sodium. We can only say that no fundamental difference in the rate of uptake of the two ions has as yet been found. It is intended, however, to repeat the experiments with an improved technique. KROGH (1937—38) has given good evidence that in frogs and other fresh water animals sodium and chlorine may be taken up independently; it would be of importance to verify this view by means of isotopic indicators and especially to find out whether the process of taking up sodium and chlorine may be regulated independently by hormones or by other means.

Experiments are going on to test the influence of the hormones from the adrenal cortex on the active uptake of ions, and later it is planned to test other physiologically active substances. It is our hope that the knowledge gained along this line will be helpful not only to throw some light on the osmoregulation, but that it also will further the study of the relation between the different organs showing an active transport of ions. In vertebrates, active transport of sodium and chlorine is known to occur in the gut (cf. VISSCHER et al. 1944), and the kidney tubules, and in water living forms through the skin or the gills. Moreover, an active out-transport of sodium seems necessary in striated muscle and, probably, in most types of cells (KROGH 1946).

One way of testing the relation or non-relation between these mechanisms would be to study their reaction to physiologically active substances known to act on one of them.

However, much work has to be done before we can hope to approach these interesting relationships. Even as regards the influence of the neurohypophyseal hormones on the transport of ions, the present study represents little more than the presentation of the problems, whereas such important items as the dependence between dosis and reaction and the effect of removal of the pituitary will have to be treated later.

### Summary.

1) The active uptake of sodium from dilute solutions of sodium chloride by the axolotl has been studied with radio-sodium. The mean rate of uptake was found to be  $68.7 \mu\text{equiv. per day}$ . The daily excretion of starving animals was found to be somewhat higher, viz.  $94.6 \mu\text{equiv.}$

2) Injection of Insipidin, a pressor preparation from the neurohypophysis, induces a net uptake of sodium and chlorine lasting for several days.

Studies with radio-sodium revealed that the net uptake is due to an increased active uptake (the increase amounting to 200 % of the normal sodium uptake), whereas the excretion is not depressed. Indeed, an increase in the excretion lasting for several hours after the injection was always seen.

3) The oxytocic principle, represented by the preparation Pitupartin, induces a rapid net loss of sodium and chlorine lasting for 2—5 days. This loss was found to be due to an increased excretion, whereas the active sodium uptake was practically unaffected, apart from a short increase in the rate of uptake immediately after the injection.

4) The injection into the animals of hypertonic sodium chloride solution calls forth an increase in the active uptake as well as in the excretion of sodium. After a few hours, both uptake and excretion as a rule returned to the normal level. The mere introduction of the injection needle produced a quite similar reaction. This pricking effect is easily distinguished from the pressor principle reaction on account of the short duration of the former.

5) The results are discussed and it is concluded that the transient increase both in excretion after Insipidin and in uptake after Pitupartin may be due to the pricking effect, so that the pressor principle may be assumed to act upon the active salt uptake only, whereas the oxytocic principle mainly (or only) increases the rate of the salt excretion.

The authors wish to express their sincere thanks to Professor A. KROGH for his active interest in our work with radioactive indicators and for the inspiration he has given with his studies of the ion transport and the permeability of the cells.

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The axolotls were most kindly put at our disposal by Mr. J. GAD-ANDRESEN, Director of the Gea Ltd. Mr. SCHMIDT-JENSEN, the State Veterinary Serum Institute, has given valuable advice concerning the treatment of the animals.

The radio-sodium and radio-chlorine were prepared in the Copenhagen cyclotron. We wish to extend our gratitude in the first place to Professor NIELS BOHR for his very great kindness and never failing support and, moreover, to cand. mag. O. LASSEN for his readiness to provide us with numerous radioactive samples.

The firms ALFRED BENZON and MEDICINALCO, Copenhagen, have furthered our work by generously putting the hormone preparations at our disposal.

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From the Physiological Institute, Lund.

## On the Influence of Muscular Work on the Citric Acid Content of Human Bloodserum.

By

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In most of the investigations carried out by Thunberg's enzymatic method for determination of the Ci content in biological material (THUNBERG 1929, 1933), the theoretical basis for the method has only been mentioned parenthetically. As a change, moreover, of late has taken place in our conception of this basis, a more precise statement for it would now be appropriate.

The decolourating effect on the methylene blue (= Mb), which under anaerobic conditions appears when Mb and Ci (Ci = citric acid or citrate) is added to a cucumber-seed extract, was in the beginning interpreted from the simple assumption that one had here to deal with a chemical "threebodysystem". Accordingly the system was considered being composed of only a donor substance (citric acid), a specific dehydrogenase (citrico-dehydrogenase), and an unspecific hydrogen acceptor (Mb). The process taking place in the system was assumed to consist of activation of a hydrogen group in the citric acid under the influence of the citrico-dehydrogenase and the reaction of the activated hydrogen group with Mb, which thereby under decolouration is transformed into leuko-Mb.

This decolouration has, however, proved to be the result of a more complicated series of processes than first believed.

The possibility and necessity of a new interpretation of the Mb-decolouration, when Ci is added to the cucumber-seed extract, was presented by WAGNER-JAUREGG's and RAUEN's demonstra-

tion that isocitric acid has just as strong, if not stronger decolourating effect than Qi on the cucumber-seed extract to which Mb is added. In contrast to Ci the isocitric acid has a configuration that without difficulty permits the stating of a formula for its dehydrogenation. According to the formula the isocitric acid is transformed into  $\alpha$ -ketoglutaric acid, a substance whose character of metabolite has long been asserted. Finally, in the interpretation of the decolouration process one had to pay attention to that there is no dehydrogenase, active on citric acid, in a cucumber-seed extract.

The problem about the hereby working mechanism has been solved by KNOOP, MARTIUS, JACOBSON, KREBS and JOHNSON. (But see also OCHOA 1945). The primary effect is due to the presence in the cucumber-seed extract of an enzyme with the power of transforming Ci into isocitric acid. The enzyme has received the name aconitase because the transformation takes place under formation of aconitic acid (cis-aconitic acid). It is a water-transporting enzyme, analogous to the fumarase discovered by BATTELLI and STERN. Under the influence of the enzyme an elimination of a water molecule from the citric acid with formation of aconitic acid takes place first, whereupon water is again introduced to the aconitic acid, but on another part of it. Isocitric acid, an isomere to citric acid, is formed in this way. If any of the components of the system citric acid — isocitric acid — aconitic acid in a solution is under the influence of aconitase, the others are formed too and an equilibrium is established in which citric acid is assumed to be present with about 80 per cent, cisaconitic acid with 4 per cent, and isocitric acid with 16 per cent. The result of this enzyme effect on citric acid is a corresponding formation of isocitric acid. As the latter is dehydrogenated, additional isocitric acid is formed in proportion. A determination of the isocitric acid formation gives therefore simultaneously a value of the actual quantity of citric acid.

One may consequently from a certain point of view consider the citric acid in biological material as a reserve store that is mobilized in the form of isocitric acid and as such is consumed.

It is now very near at hand to believe that the simple scheme of a biological threebodysystem, which has originally been employed in order to explain the Mb-decolouration, could still be used, if only isocitric acid is introduced as donor substance instead of citric acid. Under the influence of an isocitrico-dehydro-

genase a hydrogen group from the isocitric acid then should be transferred to Mb, which in this way should be decolourated.

But here, too, the conditions were found to be more complicated. Further studies have proved that this new system, too, for the Mb-decolouration must be completed by co-ferment Warburg and by an enzyme belonging to the group of yellow enzymes (the flavinenzymes), probably represented by v. Euler's diaphorase or a substance closely related to the latter. These substances direct the metabolism in the following way.

Under the influence of the isocitric acid-dehydrogenase a hydrogen group in the isocitric acid is mobilized and is forced over to co-ferment Warburg under the formation of the latter's reduction form. This form of co-ferment Warburg is now attacked by the flavinenzyme mentioned, which activates one of the hydrogen groups of the co-ferment. Only in this way does the hydrogen obtain the power to react with Mb, which is then decolourated and changed into its leukoform..

This way of explaining the transfer of the hydrogen group represents the opinion of M. DIXON, and accordingly attributes to co-ferment Warburg the character of a specific hydrogen-acceptor. But it is also possible to regard the isocitric-acid-dehydrogenase as an apoferment, which does not obtain the character of a holo-dehydrogenase and consequently the ability of activating hydrogen until the co-ferment is added.

Whether one looks upon co-ferment Warburg as a specific hydrogen-acceptor or as a complement to an apodehydrogenase, a formation of leukomethyleneblue takes place to the same degree as citric acid is added. And the amount decolourized methylene blue can be used as a measure of the amount of citric acid added to the system.

In Thunberg's Mb-method a citric acid determination is in practice built on the setting up of two curves. One, the "standard curve", is constituted by the decolouration times in their dependence upon added, known quantities of Ci, the other, the "x-curve" is formed by the decolouration times obtained by varying quantities of x-solution. As equal decolouration times represent equal quantities of Ci, the Ci-values for the different points on the x-curve are obtained by the projection of these points on the standard curve.

By means of this method useful for very small quantities of citrate one has discovered citric acid in several substrates which

have been inaccessible to not so sensitive methods. Thus it is now known that practically all animal fluids and tissues have a more or less high content of citric acid.

When SJÖSTRÖM in 1937 launched the citrate-analysis of human blood-serum as a diagnostic method for some liver diseases this aroused quite a lot of interest in the citric acid metabolism among clinicians in Scandinavia. In the course of investigations in this way evoked, the method was made more sensitive, and a survey over its sources of error and the means to eliminate them was begun. Uncertainty still prevails, however, on different points. This holds *e. g.* good for the dependence on muscle work. Certain observations exist indeed. LEHMANN found muscular work increase the citrate content of serum but on the contrary LÖVGREN found a marked reduction of it.

The investigation, the result of which is set out in this paper, aims at carefully find out this influence and in this way get the necessary basis for fixing the conditions, when one has to draw blood-samples for diagnostic use.

The investigation aims also to give an answer to the question which citrate values may be considered normal for a healthy person under certain standard conditions. — About this question are not quite corresponding opinions. Different authors find values between 15—28  $\gamma$  Ci per ml serum. The Ci-value refers then to the acid with crystal water. (Mol.-W. 210,13. For reduction to acid free from crystal water one must multiply with 0,9143.).

Thunberg's method is, however, positive also for iso-citric acid. It would under such circumstances be more just to substitute the expression "citric acid" with some designation which summarized the both substances directly or indirectly responsible. It is, however, uncertain if isocitric acid exists in blood-serum. If there is no isocitric acid in the serum, the pentabromacetone method which does not react on isocitric acid ought to give lower values for the "citric acid" content in serum than the enzymatic method.

But such a difference between the result of the enzymatic method and the pentabromacetone method has not yet been proved with certainty. The enzymes, which participate in the forming of the equilibrium system (citric acid — isoconitic acid — isocitric acid) are intracellular enzymes, and there is no proof that a corresponding equilibrium system exists in blood serum. Acknowledging the desirableness that the different elements of the system

are kept apart when determined one may consider it fully authorized for the present to denominate them "citric acid".

The cucumber-seed extract which is used as reagent on citric acid contains also an enzyme which is specific for ethyl-alcohol, an "alcohol-dehydrogenase" (THUNBERG). Without being able to compete with the decolourating effect on Mb of citric acid dehydrogenase it asserts itself by an alcohol content in the blood of 1:1000, and it will in this case add its decolourating effect to that of the citric acid dehydrogenase. If one wants to obtain a clean citric acid value, the consumption of alcohol ought then to be avoided by the subjects.

Serum has been obtained of blood taken by puncture of the cubital veins, because this blood is considered to correspond as much as possible with the arterial blood.

In a preparatory investigation the ci-values were stated of 6 subjects, who had to appear in the laboratory early in the morning and before having taken any food. Here they had to lie and rest for one hour before a blood sample was drawn. It appeared that of these 6 subjects, 5 had Ci-values between 27 and 33, that is to say in the upper edge of or above what in the laboratory had usually been regarded as normal. Only one subject had a clear "normal value". The mean value for these 6 samples was 29.

Now one had to take in consideration the possibility that the subjects already through the relatively insignificant "muscle-work" of getting up, dressing, walking or cycling to the institution etc. had reached a citrate-value which had not returned to the rest-value through an hour's rest. A certain psychic strain on the subjects may have had an effect here too.

In order to eliminate these sources of error the experimental conditions were altered in the following way: A first blood sample (A) was drawn from the subject early in the morning while he was still in bed. Then the subject had to get up and dress and walk or cycle to the laboratory, a distance of about 2 km., where a second blood sample (B) was drawn about 1 hour after sample A. Then the subject had to lie down and rest for 1—2 hours before the third and last sample (C) was drawn.

The experiments were carried out on an empty stomach on subjectively healthy persons. On the whole 19 experiments have been carried out. The experiments have been repeated a few times on the same subject with an interval of a couple of weeks. The subjects have represented ages between 9 and 73 years. 14 of the experiments have been carried out on women and 5 on men (one of these a 9-year-old boy). The pulse frequency in the A experiments (the rest samples) was between 60 and 76, in the B experiments (work experiments) between 80 and 160, and in the C experiments (the final values) between 58 and 88.

In the table the information concerning age, sex and pulse frequency

has been omitted because no correlation between these values and the Ci-values has appeared distinctly, possibly owing to the fact that the relatively small number of experiments have made a statistic treatment impossible.

In the table the experiments repeated on the same person have been inserted first and have been brought together in groups in chronological order. Then all the other experiments have been inserted, also in chronological order. For each separate experiment with a few exceptions the A-value, B-value and C-value representing resp. rest-value, working value and (hypothetically) restitution-value are reported.

Table.

*γ ci/ml. serum.*

		Sample A	Sample B	Sample C.
14/3	S. K. ....	28	38	36
20/3	S. K. ....	26	29	27
16/4	S. K. ....	27	32	—
28/2	I. A. ....	28	—	32
16/3	I. A. ....	24	29	27
9/4	I. A. ....	25	28	28
19/3	I. L. ....	27	24	25
3/4	I. L. ....	27	26	30
23/4	I. L. ....	32	31	33
15/4	B. L. ....	29	40	24
27/4	B. L. ....	31	41	34
3/5	B. L. ....	29	30	26
13/2	K. O. ....	29	35	35
16/2	S. E. ....	33	34	36
20/2	J. O. ....	25	30	33
8/3	E. L. ....	23	28	29
10/4	P. A. ....	28	36	33
11/4	H. O. ....	29	32	31
12/4	T. T. ....	31	34	33
mean value		28 ± 0.60	32 ± 1.07	31 ± 0.87

The table shows that out of the 19 experiments 11 gave initial values of 28 or below. Of the remaining 8, 4 gave values between 28 and 30, and 4 above 30. Subjects who are accustomed to this investigation have on an average given somewhat lower initial values than others. The mean value of all 19 initial samples is 28.

In spite of the fact that all the A-samples in these series were taken in the morning and that they accordingly were aimed at

representing the rest-values, the mean value is higher than those of the previous investigators.

That it is easier to produce a definite rest-value from hospital patients confined to the bed than from polyclinical patients is very probable, as the bed-patients after a short time get accustomed to the different samples. They are neither physically nor psychically disturbed as much as less trained subjects by the treatment which a puncture of a vein implies, how insignificant it is. This agrees with statements from clinical quarters. — If the  $C_i$ -value is not influenced by endogeneous circumstances it usually gets lower on patients who have been lying in hospital for some time than on those who have recently arrived there.

The circumstances, when blood-sample B was drawn, correspond on the whole with the conditions, when the samples are taken polyclinically without any preceding rest period. In 15 samples out of 18 we got here an increase of the citrate-content from sample A. The increase lies, however, in 3 cases within the limits of the method's error. 3 samples have given an insignificant diminution, which, however, also lies within the limits of this error. The mean value for the B-sample was 32. As is evident, however, from the calculated mean error, the average increase of the  $C_i$ -value from the A-series to the B-series is statistically significant.

The sample C drawn after rest has in the various experiments given various results. In 9 samples the value has fallen below sample B, thereof in one case, with a high B-value, even below the value of sample A. In 6 of these the fall has been insignificant and has lain within the limits of error. In 6 cases — yet only in one case surely — an additional increase has been obtained in spite of the rest. In a few cases no change had occurred or was noted as uncertain. The mean value for the C-sample was 31.

The conception had already from other quarters been stated that the  $C_i$ -value in serum rises under the influence of muscular work. The conception in question is strongly sustained by the here stated experiments. They are directly adapted to this question, and they have been carried out under consideration of known sources of error.

The increase of the citrate content in the blood-serum under influence of muscular work seems to be of biological interest. The acid is formed as an intermediary product in the carbohydrate metabolism. But also in the metabolism of protein and fat a citric formation seems to take place.

This investigation intends also to give an account, if a certain Ci-value may be considered normal or pathological. When the blood-sample is drawn in bed before rising under "standard conditions" C-values higher than 30  $\gamma$  are seldom and may be looked upon as pathological. In liver diseases there then is good reason to diagnose hepatitis. If the blood-sample is drawn after beginning of the day's work, as on an out-patient's department at a hospital, one has to be very careful using the Ci-values.

### Summary.

By THUNBERG's enzymatic method the citrate content of serum has been determined on human blood from the cubital veins of healthy subjects. The blood samples have been drawn, an A-sample before rising, a B-sample after the subject having performed a certain muscular work and a C-sample following a 1—2 hour's rest (bed-site) after the B-sample. 19 series have been carried out in all. For the 19 A-samples the minimum-value, mean-value and maximum-value were 23, 28, and 33  $\gamma$  citric acid per ml. serum. The corresponding values for the B- and C-samples were 24, 32, 41 and 24, 31, 36. The Ci-value is increased through muscular work. Using the Ci-value of serum for diagnosis one has to take the blood-sample before rising. As upper limit for healthy subjects one can fix 30  $\gamma$  Ci. Of 19 values only 3 are a little higher. When the bloodsamples are taken after the beginning of the day's work as on an out-patient's department of a hospital the Ci-values are more variable, and have to be estimated with reservation.

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## The Biological System Calcium—Phosphate—Citrate.

By

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Since some decade it has been known that citric acid enters as a component in practically all the fluids and tissues of the animal organism (THUNBERG, DICKENS). The explanation for this lies undoubtedly in the citric acid's character of a metabolite which is formed in connection with the carbohydrate metabolism (KREBS & JOHNSON). It is, however, not more than 6 years ago that DICKENS discovered that citric acid enters also in a considerable amount in bone substance. Citric acid was also found in calcium concrements of different kinds (MÅRTENSSON 1941, THUNBERG 1941).

This last-mentioned fact must have something to do with a precipitation of citrate in a slightly soluble form, and one asks oneself which this form might be.

It is near at hand to think of tricalciumcitrate  $(C_6H_5O_7)_2Ca_3 + 4H_2O$ .

Tricalciumcitrate shows interesting conditions of solubility. It is relatively easily soluble in cold water. On the other hand it is slightly soluble at a higher temperature, a fact already observed by SCHEELE, the discoverer of the acid, and which he employs for the characterising and isolating of the acid. Thus, according to WARRINGTON, one part of the crystalline salt is dissolved at 14° C in 1180 parts of water, and at 90° in 1730 parts. The amorphous salt is more easily dissolved.

A new possibility for explaining the origin of slightly soluble citrate combinations has been brought forward by KUYPER 1938,

who found that citrate is quantitatively precipitated from a solution which besides citrate contains phosphate-ions and calcium-ions in a suitable concentration. In the precipitate produced in this way citric acid will not be found as calcium citrate, but in a somewhat complex form. It is possible that one here has to deal with a substance, in which calcium as well as phosphoric acid and citric acid enter.

If the latter assumption is right phosphoric acid ought besides calcium and citric acid to enter not only into bone-substance, what one already knows is the case, but also in such calcium concrements that have been found to contain citric acid.

In order to verify the correctness of this assumption a series of analyses have been undertaken. They attach themselves to analyses which have already been carried out in another connection by THUNBERG (1941), and which aimed at an examination of the appearance of citric acid in calcium concrements of different kinds. Citric acid has thus been proved in a couple of now obsolete pharmaca. To these belong *Lapis cancri*, concrements which are formed in pockets of the crayfish's stomach during the changing of the shells and which is decomposed in connection with the formation of the new shell. To these belongs too *Cornu cervi rasum*, chips of horn from deer or cattle. From earlier investigations by THUNBERG (1941) it was also known, that eggshell contains citric acid.

The analyses were now repeated and extended to include the presence of phosphata. The investigations included also oyster shell and coral. As DICKENS and THUNBERG (1941) found, also keratin contains citric acid. Analysis on citric acid, calcium and phosphor were made on this material too.

Table.

	Citric acid g/100 g	Phosphor g/100 g	Calcium g/100 g
<i>Cornu cervi rasum</i> (Pharm. Suec. Ed. VII) .....	1.560 1.280 1.280	9.0  9.5	25.6  25.6
<i>Lapis cancri</i> (Pharm. Suec. Ed. VI) .....	0.520	2.05	29.2
<i>Birds' eggs, shells</i>			
<i>Gallus domestica</i> .....	0.15	0.154	33.3
<i>Somateria mollissima</i> .....	0.13	0.15	33.3
<i>Fulica atra</i> .....	0.29	0.15	32.8

	Citric acid g/100 g	Phosphor g/100 g	Calcium g/100 g
<i>Crocodile egg, shell</i>			
Croc. Johnstoni .....	0.055	0.048	23.6
<i>Marine animals</i>			
Oyster shell .....	0.017	0.019	32.8
White coral .....	0.024—0.013	0.007	35.2
<i>Keratin substance</i>			
Human hair, blond .....	0.017—0.012	0.003	0.21
		0.003	
white .....	0.023	0.0018	
Horse Hoof .....	0.045	0.004	0.12

The table shows that citric acid, calcium and phosphor go together as components in the substances which were examined. They do not contain calcium and citric acid alone, but also phosphor. All these 3 substances have been found also in the examined keratin substances, be it that both the citric acid content as well as the phosphorous content is small. From KUYPER's investigations 1938 it appeared that citric acid does not precipitate quantitatively from a solution which only contains a very small amount of calcium or phosphor. This agrees very well with the low citric acid content in the keratin substances which of course also contain but very small quantities of calcium and phosphor. The table shows also that in formations with high citric acid content the phosphor content is high too.

The observations published here seem not to be without value for a hypothesis which ANNERSTEN has brought forward concerning the chemical background of the ossification. He thinks that an ossification hormone plays a certain part in the formation of bone substance. It does not seem improbable that what ANNERSTEN interpreted as an ossification hormone is a citrate indeed. Out of certain living tissues he produced an extract which in several respects seems to have the quality of citric acid. He has come across the hormonal factor in the urine, and the urine contains considerable quantities of citric acid. The active factor is soluble in alcohol and ether. Citric acid is also soluble in alcohol and ether, to be sure only in the shape of a free acid. One has come across the hormonal factor also in blood and muscle tissue, just as is the case with citric acid.

*Methodic.* Citric acid has been stated according to PUCHER, SHERMAN & VICKERY. As a means of decoloration for the surplus of perman-

ganate  $\text{H}_2\text{O}_2$  was employed. The coloured solution obtained by sodium sulphide was absorbed in acetone.

The analysis of phosphor was carried out colorimetrically according to FISKE & SUBBAROW.

The calcium analyses were carried out according to KRAMER & TISDALL.

These analyses were carried out in the winter of 1944—1945. At this time KUYPER had not yet published his investigations about the bone formation problem.

### Summary.

In the hitherto investigated concrements which contain calcium and citrate the author has now stated the presence also of phosphoric acid. It seems probable that the slight solubility of a complex containing Ca, citrate and phosphate here plays a part.

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## About the Pyrogen Activity of Dialyzed Casein Digest.

By

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Ever since ELMAN and WEINER (1939) made the first intravenous injection of amino acids in man, the intravenous administration of amino acids has become more and more common in surgical as well as in medical practice. In their first cases ELMAN and WEINER used amino acids prepared from casein hydrolyzed with sulphuric acid. It was because tryptophane is destroyed during acid hydrolysis that this amino acid was added. In later experiments ELMAN (1940) as well as GARDNER and TRENT (1942), LANDESMAN and WEINSTEIN (1941), WHITE and WEINSTEIN (1945), have used a product of amino acids called Amigen, which is prepared by enzymatic hydrolysis of casein.

In making casein digests of that kind, it is rather difficult to get them free from pyrogens. ZITTLE, DEVLIN, RODNEY and WELCKE (1945) have described a method for removing pyrogens from acid protein hydrolysates by means of Seitz filtration. Results showed that partial hydrolysates (enzymatic) could not be freed from pyrogens by Seitz filtration. In their enzymatic casein hydrolysates they found very large amounts of pyrogens. The investigation reported below, however, shows that it is possible to make enzymatic casein hydrolysates which are free from pyrogens. The method has been described elsewhere (WRETLIND 1944) and consists briefly in hydrolyzing pure casein by purified trypsin and erepsin. When the hydrolysis is ready, the hydrolys-

ates are dialyzed in order to maintain the largest possible content of amino acids and low molecular peptides. This casein digest, which has been used in Scandinavia under the name of Aminosol,<sup>1</sup> has shown good properties in intravenous administration (LIDSTRÖM 1944 and WRETLIND 1945). It consists of 67 % of free amino acids and in a previous investigation (WRETLIND 1946) it has been found that practically all amino acids are partly in free form. In order to show that this amino acids preparation has a very small content of pyrogens, the following experiment was performed.

### Experimental.

The determination of pyrogens is usually performed on rabbits by measuring the temperature elevation after the injection of the solution. SEIBERT and MENDEL (1932) have shown that the temperature in rabbits has a daily variation of  $0.54^{\circ}\text{C}$ . Thus a rise of more than  $0.54^{\circ}\text{C}$ . shows the presence of pyrogens. The *U. S. Pharmacopæia XII* (1942) has the following test of pyrogenfree solutions. An injection of 10 ml of the solution per kg rabbit in 5 rabbits should not give a temperature rise of more than  $0.6^{\circ}\text{C}$ . if the solution should be called pyrogenfree. The temperature is taken immediately before and 1, 2 and 3 hours after the injection. SEIBERT and MENDEL (l. c.) have shown that the temperature elevation generally sets in during the first hours after the injection. With regard to the amino acid solution used here, these findings have been verified by the author and thus the temperature has been taken only during the first 3 hours after the injection.

Rabbits weighing 1750 to 2250 g were used. The amount of the injected amino acid solution was in every case 20 ml. The injected preparation was a solution of 3.3 % Aminosol in pyrogenfree redistilled water. In some cases 5 % glucose were added to the solution. The temperature was measured in the rectum with a standardized thermometer inserted beyond the sphincter for three minutes. The rabbits were kept in their light, wellventilated cages and were allowed to eat as usual. During the temperature measuring and injections the rabbits were gently lifted from their cages in order not to disturb them, which might elevate the temperature. The temperature was taken immediately before the injection, which was done within 2—3 minutes in an ear vein, and then 1, 2 and 3 hours after the injection.

The values are given in table I. The table shows that the temperature in general increased one hour after the injection and then slowly decreased in the following 2 hours. The number of the Aminosol solution in the table relates to the different sets of preparation.

<sup>1</sup> Aminosol is prepared by Vitrum, Stockholm.

Table I.

Aminosol solution No.	T e m p e r a t u r e ° C			
	before injection	after 1 hour	after 2 hours	after 3 hours
532 .....	38.6	39.1	38.9	38.5
533 .....	38.3	38.8	38.8	38.7
535 .....	38.7	39.0	39.5	39.1
536 .....	38.3	38.8	38.7	38.8
544 .....	38.3	38.8	38.5	38.6
546 .....	37.7	38.2	38.8	38.5
547 .....	38.4	38.7	39.1	38.7
547 .....	38.4	38.4	38.7	38.6
553 .....	38.6	39.2	38.9	38.9
553 .....	38.7	39.2	39.1	38.7
554 .....	38.7	39.0	39.5	39.3
554 .....	37.9	38.6	38.5	38.7
555 .....	38.8	39.4	39.5	39.2
555 .....	39.1	39.3	39.1	38.8
557 .....	38.2	39.5	39.3	39.1
557 .....	38.7	39.1	38.9	39.0
558 .....	39.2	39.8	40.0	39.8
558 .....	39.0	39.1	39.2	39.0
559 .....	39.0	39.4	39.2	38.9
559 .....	39.1	39.6	39.4	39.4
560 .....	39.2	39.6	39.6	39.3
560 .....	39.2	39.3	39.4	39.3
562 .....	38.7	39.2	39.4	39.7
562 .....	38.8	39.6	39.7	39.7
563 .....	39.0	39.4	39.0	38.8
563 .....	38.0	39.5	39.2	39.3
564 <sup>1</sup> .....	38.8	39.0	38.8	38.6
564 <sup>1</sup> .....	39.0	39.2	39.4	39.1
567 .....	39.1	39.2	39.3	39.3
567 .....	39.1	39.0	39.0	39.0
1000 <sup>1</sup> .....	38.9	39.5	39.4	39.5
1000 <sup>1</sup> .....	39.3	40.0	39.6	39.3
1001 <sup>1</sup> .....	38.7	38.8	38.6	38.7
1001 <sup>1</sup> .....	39.0	39.2	39.3	39.1
Average:	38.75	39.16	39.16	39.03

From the figures of the temperatures it is evident that the temperature elevation is very seldom more than  $0.60^{\circ}$  C. The average temperature elevation is  $0.41^{\circ}$  C. after one hour and  $0.41^{\circ}$  C. and  $0.28^{\circ}$  C. after two and three hours, respectively. From this

<sup>1</sup> With 5 % glucose.



it might be concluded that this amino acid preparation contains no or merely a very minute amount of pyrogens. All these Aminosol solutions have been given also to human subjects without any fever reactions (LIDSTRÖM 1946). The rate of the intravenous injection was 300—500 ml per hour. In general, the patients received 1000 to 2000 ml a day.

### Summary.

It has been shown that a preparation of dialyzed enzymatic casein digest contains no pyrogens. The pyrogen test was performed on rabbits by measuring the temperature elevation after the injection of the casein digests.

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## Variations in the Carbon Dioxide Tension in the Arterial Blood and the Electrocardiogram in Man.<sup>1</sup>

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In earlier investigations (1946) the author has shown that hyperventilation in atmospheric air brings about distinct electrocardiographic signs of myocardial hypoxaemia, and that these changes result from the reduced carbon dioxide tension in the blood. Moreover it is demonstrated that these changes are not due to the impeded dissociation of the oxyhaemoglobin caused by the reduced carbon dioxide tension in the blood. The tachycardia caused by the hyperventilation in atmospheric air can have no influence on the occurrence of the electrocardiographic changes, since no such changes are seen during hyperventilation in air containing 5 per cent carbon dioxide, although a pronounced tachycardia is present also in these experiments. As administration of coronary dilating agents, such as nitroglycerin, have no influence on the electrocardiographic changes it seems reasonable to conclude that constrictions of the coronary vessels can be of no essential importance for the appearance of the myocardial hypoxaemia. As the author (1945 a) has previously demonstrated that the arterial pressures undergo no changes during hyperventilation in atmospheric air, it seems reasonable to presume that the transitory

<sup>1</sup> With financial support from Kong Christian den Tiendes Fond.

myocardial hypoxaemia during hyperventilation is conditioned in the first instance on a reduced flowing of blood through the coronary vessels on account of a compression of the latter, due to an increased intramyocardial tension, since HENDERSON (1908) has proved, in convincing experiments on dogs, that the volume of the heart decreases remarkably during hyperventilation in such a way that the diastolic volume is reduced first and then the systolic volume, after which the heart finally stops in systolic contraction.

These previously reported investigations elucidate, however, only the qualitative conditions. The investigations to be reported here should throw light on the question as to which carbon dioxide tensions cause the electrocardiographic changes to occur.

### Methods.

The report here presented is based on 10 experiments carried out on 2 circulation-sound persons aged 30 and 42 years respectively.

The experiments were carried out in the morning and were undertaken after the persons, who were fasting, had lain in rest for one hour in the laboratory. After this period of rest 2 electrocardiograms were recorded at intervals of 5 minutes in order to make sure that the electrocardiogram presented no spontaneous changes of any note. Next came the recording during the hyperventilation, which lasted from 3 to 8 minutes.

In the case of one subject only the 3 usual extremital leads were recorded, while in that of the other also the precordial lead 4 R was applied.

The various leads were recorded synchronously. The rate was calculated on the basis of the time elapsing between 8 successive R-waves. The electrocardiograph was at all tracings adjusted in such a manner that a difference of tension of 1 millivolt brought about a deflection of 10 mm. The measurement of the heights of the individual waves was carried out according to LARSEN's (1936) method, at which the straight line tangent to the lower border of the curve on the segment between the end of the P-wave and the beginning of the initial complex, is used as the iso-electric level. This line does not always run a horizontal course, but may be either slightly ascending or descending. In such cases the straight line tangent to the curve at the beginning of the initial complex is used as the iso-electric level. This line does not always coincide with the actual iso-electric level. The latter must be the straight line tangent to the lower border of the curve between the end of the T-wave, or sometimes the U-wave, and the beginning of the P-wave, as the electrocardiographic curve between the end of the P-wave and the beginning of the initial complex will sometimes run a few tenths mm below the T-P segment. The stated heights of the individual waves have, like the indicated times, been calculated as the average of

the respective values of 5 successive complexes. Alveolar air tests were made immediately before and after each electrocardiogram in order thus to determine the carbon dioxide tension in the arterial blood.

A slightly modified form of HALDANE and PRIESTLEY's method was applied for the determination of the composition of the alveolar air.

As voluntary hyperventilation is rather difficult to carry through for several minutes together even for a trained subject, breathing through a mouthpiece and with noseclips was performed only for the short periods during which alveolar air samples were taken. For this reason a short, wide tube was applied as mouthpiece. When the subject had become familiar with the technique through a few preliminary experiments, it was easy to make her take the piece into the mouth and make the lips close tightly round it. The nasal respiration was prevented in the simple manner that an assisting person pressed the nostrils together digitally during the expiration, within which period the alveolar air samples were taken.

The alveolar air samples were made at the end of a deep expiration, and a number of experiments of alveolar air analysis were made at intervals of 30 seconds in order to make sure that practically identical values were obtained for the composition of the alveolar air.

### Results.

Each of the subjects presented complete accordance between the variations in the composition of the alveolar air and the variations in the electrocardiograms at the different experiments, therefore only a single experiment from each subject shall be mentioned in order to show the dependence of the electrocardiographic changes on the carbon dioxide tension.

Fig. 1 illustrates 3 sets of electrocardiograms from a 30-year-old woman with a normal circulation. The first set was recorded before the hyperventilation and shows perfectly normal conditions. The second set was recorded after 2 minutes of hyperventilation and shows marked changes. The third set, which was recorded after another 2 minutes of hyperventilation, displays pronounced signs of coronary insufficiency. The composition of the alveolar air, at the moment the electrocardiograms shown here were recorded, appears from table 1, in which the electrocardiographic characteristics are likewise indicated. It appears from the table that there are found distinct electrocardiographic signs of coronary insufficiency at a carbon dioxide tension of 20 mm Hg. Thus there is seen a pronounced depression of the S—T intervals in all three extremal leads. At a carbonic acid tension of 16 to 17 mm Hg in the alveolar air, and thus also in the arterial blood, the changes

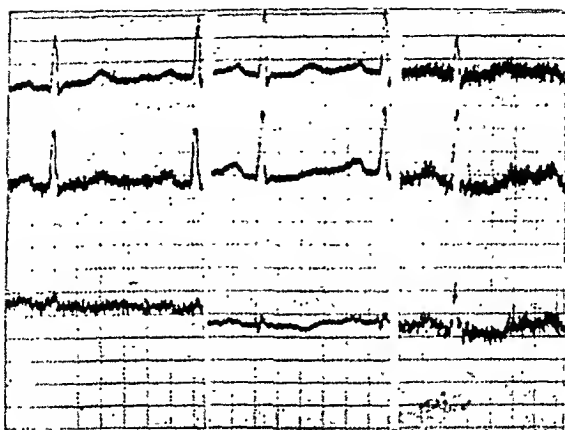


Fig. 1. Electrocardiograms from subject No. 1.  
1st set of eeg. recorded during normal respiration, 2nd and 3rd sets after 2 and 4 minutes respectively of hyperventilation in atmospheric air.

Table 1.

*Characteristics of electrocardiogram.*

Alveolar air					Time indicated in seconds, height of waves in mm														
O <sub>2</sub>		CO <sub>2</sub>		RQ															
%	mm Hg	%	mm Hg			Rate	P-Q	Q-T	P <sub>1</sub>	P <sub>2</sub>	P <sub>3</sub>	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	S-T <sub>1</sub>	S-T <sub>2</sub>	S-T <sub>3</sub>	T <sub>1</sub>	T <sub>2</sub>
15,75	112	5,10	36	0,97	93	0,14	0,30	1	2	1	11	12	1	0	0	0	2½	2	-1
15,72	112	5,47	39	1,04															
18,78	134	2,75	20	1,27	111	0,14	0,30	1½	2	1	14	14	0	-1	-1½	-½	2	½	-1½
18,10	128	2,50	17,8	0,87	111	0,14	0,28	1	2	1	7	15	8	-1	-2½	-1½	1½	0	0
17,89	127	2,17	15,5	0,70															

are far more pronounced. The depression of the S—T intervals has become deeper, and T<sub>2</sub>, which at 20 mm Hg acid tension was low, but still positive, has now become iso-electric.

A number of electrocardiograms were recorded at carbon dioxide tensions lying between 30 and 23 mm Hg; but electrocardiographic changes were demonstrated in none of these cases. Accordingly it seems safe to conclude that the electrocardiographic changes occur when the carbon dioxide tension has fallen, through hyperventilation, to about 20 mm Hg or less, in other words when the tension has fallen to about half of the normal.

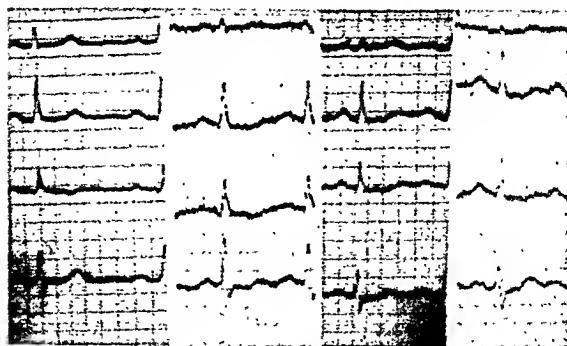


Fig 2. Electrocardiograms from subject No. 2.  
1st set of eeg. recorded during normal respiration, 2nd and 4th sets, from the same experiment, recorded after 2 and 5 minutes respectively of hyperventilation.  
3rd set, derived from a similar experiment, recorded after 2 minutes of hyperventilation.

Table 2.  
*Characteristics of electrocardiogram.*

Alveolar air					Time indicated in seconds, height of waves in mm																		
O <sub>2</sub>		CO <sub>2</sub>		RQ																			
%	mm Hg	%	mm Hg		Rate	P-Q	Q-T	P <sub>1</sub>	P <sub>2</sub>	P <sub>3</sub>	P <sub>4</sub> R	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub> R	S-T <sub>1</sub>	S-T <sub>2</sub>	S-T <sub>3</sub>	S-T <sub>4</sub> R	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>	T <sub>4</sub> R
14.38	102	4.82	34	0.85	75	0.16	0.34	1.2	1	1	4	11	5½	9	0	0	0	0	2	2	-1	3½	
14.41	102	4.32	31	0.66																			
18.70	132	2.78	19.2	1.20	121	0.14	0.30	1.1½	½	1	2	11	9	12	0	-1½	-1	-1½	0	0	0	1	
18.25	129	2.56	18.0	0.93	120	0.14	0.30	1.2	1	1	3	10	6	8	0	-1½	-1	-1½	1	0	0	1½	
18.49	131	2.33	16.4	0.94																			
18.78	133	2.10	14.8	0.97	130	0.14	0.28	½	2	1½	1	2	10	9	6	0	-2	-1½	-2	½	0	0	1½
18.58	132	2.10	14.8	0.89																			

Fig. 2 illustrates 4 sets of electrocardiograms from a 42-year-old woman with a normal circulation. Nos. 1, 2 and 4 are derived from one experiment, and No. 3 from another. The first set was recorded during normal respiration and shows perfectly normal conditions. The second set was recorded after 2 minutes of hyperventilation, and the fourth set after 5 minutes of hyperventilation. The third set which, as just mentioned, is from another experiment, was likewise recorded after 2 minutes of hyperventilation. It has been included here to illustrate the constancy of the changes

described from one experiment to the other. The composition of the alveolar air at the points of time at which the different electrocardiograms were recorded appears from table 2, which likewise shows the characteristics of the different electrocardiograms. This subject proved to present the same changes as the former.

Thus the electrocardiographic tracings during voluntary hyperventilation in atmospheric air have shown that the following changes will occur, when the carbon dioxide tension in arterial blood falls to ab. 20 mm Hg:

- 1) sinus tachycardia,
- 2) a slight reduction of the time of conduction,
- 3) a shortening of the electric systole duration, so that the latter falls within the range of the normal values indicated by KAJ H. LARSEN (1941),
- 4) no indisputable change of the heights of the P-waves,
- 5) uncharacteristic changes of the heights of the R-waves,
- 6) depression of the S—T segment of altogether 3 mm or more in 2 or more leads,
- 7) iso-electric or inverted T-waves in 2 or more leads.

The investigations just mentioned show that voluntary hyperventilation in atmospheric air bring about in normal man distinct electrocardiographic changes indicative of coronary insufficiency. But there is seen no prolongation of the electric systole duration; nor should we expect to find such a prolongation after SCHULTZER and LEBEL's (1939) demonstration of the fact that the concentration of calcium ions does not decrease during attacks of hyperventilation tetany.

### Discussion.

The results from these experiments with voluntary hyperventilation in normal man show that distinct electrocardiographic signs of myocardial hypoxaemia are brought about, when the carbon dioxide tension in the alveolar air is reduced to about 20 mm Hg. These results may, in the author's opinion, be of no small practical importance for the diagnosis of coronary insufficiency.

In electrocardiographic studies after muscular work or during respiration of a mixture of air poor in oxygen it is generally assumed that an electrocardiographically demonstrated coronary insufficiency is an unquestionable sign of a, possibly latent, co-

ronary sclerosis. This can, however, hardly be sanctioned as a general rule applicable to all cases, for it is a well-known fact that untrained individuals hyperventilate during muscular work so that accordingly there should be a possibility that electrocardiographic changes, if present, might have been brought about by acapnia. This fact must play a rather considerable part in the cases in which the differential diagnosis stands between coronary affection and the effort syndrome, as the author has previously (1945 b) shown that patients suffering from the effort syndrome hyperventilate so much during muscular work that there occurs a pronounced vasoconstriction of the big extremital arteries. Distinct electrocardiographic changes have in some cases been observed in apparently healthy young people at the ordinary hypoxaemia tests, where the subject is made to breathe a mixture consisting of 10 % oxygen and 90 % nitrogen. This can easily be explained as having its cause in the involuntary hyperventilation, which occurs in all individuals who breathe air that is poor in oxygen.

The facts just reported argue in favour of using the hypoxaemia test and not the working electrocardiogram for the diagnosis of latent coronary affections and of using for the hypoxaemia test a mixture of air which contains 2 or 3 % carbon dioxide in order to counteract the acapnia brought about by the hyperventilation.

### Summary.

On two subjects it is shown, that voluntary hyperventilation in atmospheric air brings about distinct electrocardiographic signs of myocardial hypoxaemia when the carbon dioxide tension in the alveolar air, and therefore also in the arterial blood, is reduced to about 20 mm Hg. It is pointed out that these findings may be of practical importance for the diagnosis of coronary insufficiency by the aid of electrocardiographic tracings after muscular work, or during respiration of a mixture of air poor in oxygen and carbon dioxide free, as these methods may cause an involuntary hyperventilation, and by this, a reduction of the carbon dioxide tension in the arterial blood.

Finally it is pointed out that these results argue in favour of using the hypoxaemia test and not the working electrocardiogram for the diagnosis of latent coronary insufficiency, and of using for



the hypoxaemia test a mixture of air containing 2 or 3 per cent carbon dioxide in order to counteract the acapnia brought about by the hyperventilation.

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# ASCORBIC ACID EXCRETION BY HUMAN KIDNEY

A STUDY ON RENAL REABSORPTIVE MECHANISM

BY

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Translated by Mrs. M. Jungstedt

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## Introduction

The study of the renal function has during the last two decades been essentially intensified, particularly thanks to the methods, introduced by Richards for determining the composition of the glomerular filtrate, and by Rehberg for determining the filtration rate. Owing to their fundamental investigations premises have been afforded *inter alia* for an increased understanding of the reabsorptive function of the tubular apparatus and especially the mechanism at glucose reabsorption.

Our possibilities of studying the glucose excretion in man, however, are hampered by the occurrence of hormonal and other factors in the organism, regulating the glucose concentration in the blood and preventing an experimental variation thereof. It would therefore for a further study of the tubular reabsorption be expedient if a substance could be found behaving as glucose in regards to excretion, its plasma concentration, however, exclusively being determined by the amount of substance administered, by the volume of distribution, and by the renal excretion. Judging from the few investigations which as yet have been published ascorbic acid seems to fulfill these requirements satisfactorily. Although chemically very active it appears to be broken down very slowly in the organism and above all not to be quantitatively influenced by uncontrollable factors.

The object of the present investigation has accordingly been to study the tubular reabsorptive rate for ascorbic acid under different experimental conditions in man. During the course of the investigation, however, results were obtained suggesting that the reabsorptive rate of the tubules had a reproducible maximal upper limit, which probably was governed by the mass of absorbent epithelium in the tubules. Hereby possibilities lay open for a functional tubule test as a complement to previously known glomerulus function test. The investigations therefore became especially directed towards the

premises for a combined renal function test, based on the excretion curve for ascorbic acid and designed to give information as well of the glomerulus function as of the reabsorptive tubule function.

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My interest in the excretion of ascorbic acid was aroused by my teacher Professor Gustaf Göthlin, M. D., the former Head of the Physiological Institution, to whom I owe a large debt of gratitude.

His successor, Professor Torsten Teorell, M. D., has given me the possibility of carrying out the present work. For this and for his support with good advice and stimulating criticism I desire to thank him sincerely.

To Henrik Enghoff, M. D., associate Professor at the Institution, I beg to express my gratitude for his valuable assistance with the statistical treatment of the material; and to Doctor Olle Hogeman for good advice concerning the inulin clearance test.

I also desire to thank my present chiefs, Gösta Bohmansson, M. D., and Olof Wilander, M. D., who have facilitated the completion of the work.

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## A short review of earlier investigations

Our present view of the function of the kidney was given its first concrete outlines by CUSHNY (1917), whose theory constituted a compromise between Ludwig's "mechanical" and Heidenhain's "active" one. Cushny adopted Ludwig's opinion that a filtration caused by hydrostatic pressure takes place in the glomeruli, and that the filtrate running down into the tubules consists of a protein-free plasma. From this preurine, water and certain substances dissolved in it are reabsorbed by an active tubular process, so that the urine receives its final composition during the passage through the tubules. Intensive experimental investigations, the results of which have been presented during more recent years, have on the whole confirmed Cushny's theory. These investigations, however, have also revealed an excretory function of the tubules.

### 1. The glomerular filtration and the clearance concept

Cushny had very weak experimental grounds for his assumption that an ultrafiltration takes place in the glomeruli; as early as 1924, however, WEARN and RICHARDS were able to show by micro-analyses of the glomerular fluid from frogs that the preurine does not contain protein, but does contain glucose and chloride, even when these substances were lacking in the bladder urine. After a series of investigations on frogs and *Necturi* it could gradually be established that the osmotic pressure, electric conductivity and the concentration of urea, inorganic phosphate, uric acid, creatinine, glucose and chloride are the same in preurine as in plasma (WALKER, 1930; WALKER and ELSOM, 1930; FREEMAN, LIVINGSTON and RICHARDS, 1930; EKEHORN, 1931; WALKER, 1933; BORDLEY and RICHARDS, 1933; BORDLEY, HENDRIX and RICHARDS, 1933; WALKER and REISINGER, 1933). For these low species, at least, the presence of a filtration in the glomeruli could be considered proved.

There is, however, no direct proof that the mechanism is the same in mammals; the general validity of the discovery of Richards and co-workers has ne-



vertheless been regarded as indirectly proved by experiments showing that, in mammals, certain substances may be excreted at an identical rate, relative to their respective plasma concentration. This applied to creatinine (in dogs) and — if the tubular reabsorption is blocked with phlorizin — to glucose, xylose, and sucrose; to ferrocyanide, and inulin (REHBERG, 1926; POULSSON, 1930; VAN SLYKE, HILLER and MILLER, 1935; CHASIS, JOLLIFFE and SMITH, 1933; SHANNON and SMITH, 1935). That at least six different substances were treated in exactly the same way by the kidney certainly suggests a passive filtration rather than an active cellular process. The ultrafiltration theory is furthermore considerably substantiated by an investigation on cats and rabbits by BAYLISS, KERRIDGE and RUSSEL (1933), where they showed that the kidney excretes protein with a molecular weight of less than about 70.000, whereas protein with a higher weight is retained. On the other hand, no experimental observations have been made arguing against the filtration theory. This theory is therefore generally accepted nowadays.

The power of the kidney to excrete a substance is expressed by the term "clearance", which was originally introduced by MÖLLER, McINTOSH and VAN SLYKE (1928). It is defined as the volume of plasma which would be required to provide the amount of the substance excreted per unit time. If  $P$  is the plasma concentration of the substance,  $U$  its urine concentration, and  $V$  the rate of the urine flow, then

$$Cl = \frac{UV}{P} \quad (1)$$

In other words, the plasma clearance of any substance is equal to its concentration ratio  $\frac{U}{P}$  multiplied by the rate of flow. For a substance neither excreted nor reabsorbed by the tubules the plasma clearance would be equal to the amount of fluid filtered through the glomeruli. A substance reabsorbed in the tubules would have a lower clearance, and a substance filtered in the glomeruli and excreted in the tubules would have a higher one.

REHBERG (1926) considered on various grounds that, after filtration in the glomeruli, creatinine does not undergo quantitative change in the tubules, and that therefore the amount excreted during a given time corresponds to that filtered during the same time. If the plasma concentration is known, it would, then, be possible to determine the amount of fluid filtered per time unit. Later, however, RICHARDS, WESTFALL and BOTT (1934), SHANNON (1935 a) and SHANNON and SMITH (1935) considered themselves to have shown that the polysaccharide inulin is completely filterable in the glomeruli, and that it is not reabsorbed or excreted in the tubules. On comparative investigations between creatinine and inulin clearance, it was seen that in e. g. dog the values are in good accordance. In man, however, inulin is said to give a somewhat lower value than creatinine,

but the difference seems from a practical point of view to be of no importance. The author will therefore not here go into detail as to the various factors, speaking for or against inulin or creatinine clearance as an absolute gauge of glomerular filtration rate in man, and refers instead to SMITH (1937) and EKEHORN (1944). The fact that inulin clearance in man is not affected by the plasma concentration, even when high (ALVING and MILLER, 1940), whereas creatinine clearance falls in rising concentrations (SHANNON, 1935 b) seems, however, at present to argue in favour of the former as the gauge of the filtration rate.

Various investigations have been done on the inulin clearance value in man. Originally, the procedure was to keep the plasma inulin concentration constant during the period of time in which the inulin excretion rate was determined, by administering a continuous intravenous injection of inulin. The result of these investigations (RICHARDS, BOTT and WESTFALL, 1938; SMITH, GOLDRING and CHASIS, 1938) showed that the clearance under physiological conditions and rest averages 120—125 ml per minute. But it was also seen that the inulin clearance was fairly constant in one and the same individual, "not only in successive 15 to 20 minute periods but week by week" (Smith et al., cit. above).

By another procedure — the determination of the excretion in sinking plasma concentrations after an intravenous injection of inulin — further investigations on the inulin clearance value have been carried out (MILLER, ALVING and RUBIN, 1940; ALVING and MILLER, 1940; BERDAL, 1940; JOSEPHSON and LINDAHL, 1943; HOGEMAN, 1943). Among these, only the latter two studies present larger series, 25 and 35 healthy subjects, respectively. With an interval of 20 to 60 minutes 3 determinations were done in all cases. A summing up gives the following values for the different periods of time:

Josephson and Lindahl ( $n = 25$ )

1 period	137 $\pm$ 6 ml per min.
2    »	143 $\pm$ 7       »
3    »	117 $\pm$ 6       »

Hogeman ( $n = 35$ )

1 period	120 $\pm$ 3       »
2    »	119 $\pm$ 2       »
3    »	121 $\pm$ 3       »

In the former material there is no significant difference between the values of the first and the second periods, while on the other hand there is a difference between the values of the third period, in comparison with the mean of the others. The authors could not with certainty account for the low values of the third period, but consider it very probable that they are occasioned by errors in technic. Neither do they make use of the values of the third period in calculating the mean values. Hogeman, on the contrary, working with methodics which *a priori* must give lower errors, finds good accordance between the values of the three periods. (It should be noted, however, that Hogeman has but an interval of about 20 minutes between his tests, while Josephson and Lindahl have 60 minutes.) In reality, however, Hogemans values in all 3 periods are so accordant, that his investigations may be said to corroborate Smith's theory, as regards the constancy of the filtration rate.

The relatively small variations found in experimental data on the filtration rate are naturally to no small extent caused by the standard errors, called forth by the complicated technic, and the kidney's power to maintain a constant filtration rate may therefore be regarded as considerable. Now, how does this take place?

Anatomical investigations (cf. CLARA, 1938) as well as physiological investigations (cf. LAMPORT, 1941) indicate that a number of physical factors influence the filtration rate: tone and resistance of afferent and efferent arteriolar, arterial and intrarenal and osmotic pressure and others; on excessive stimulus through drugs, influencing any of these, changes are also found in the filtration rate. Weaker stimuli, such as experimental variations in renal arterial pressure (SCHROEDER and STEELE, 1940) reveal, however, a certain autonomy in renal vascular bed, wherefore it is probable that the physiological factors coordinate towards the maintenance of a constant filtration rate.

Direct observations, on exposure of the glomeruli in frog (RICHARDS and SCHMIDT, 1924, and others) show that not all of the glomeruli may exhibit a free blood flow at any moment. Certain glomeruli should thus be held in reserve, to be taken into use when necessary.

These findings, however, are not directly applicable in mammals. Investigations on the tubule's capacity of reabsorbing glucose in high plasma concentrations (see below) done partly on frog (FORSTER, 1942) partly on dog and man (cf. SHANNON, 1942) indicate a fundamental difference in the manner of varying the filtration rate in these

mammals. On augmentation of the plasma concentration there is no change in the reabsorption rate either in dog or in frog. If, on the other hand, the filtration rate is augmented, in some way or another, the reabsorption rate also is augmented in frog, while no such augmentation is seen in dog. This has been given an acceptable explanation, as follows: in frog the change in filtration rate is due to the new glomeruli — and thus new nephrons — being called into use. In dog, on the other hand, all the nephrons are continuously active and an augmentation of the filtration rate is caused by a change in the filtration pressure. It thus follows that in renal function tests in dog and man, at all events on the healthy kidney, one may consider this as a single nephron.

## 2. The excretory function of the tubules

After fundamental investigations in aglomerular animals by MARSHALL (1934) on the excretory function of the tubules, Smith and co-workers showed that, in man, the clearance for certain substances — e. g. phenol red, diodrast — which were proved to have been filtered in the glomeruli, far outstripped the inulin clearance. The substances must consequently also be excreted in the tubules (GOLDRING, CLARKE and SMITH, 1936; SMITH, GOLDRING and CHASIS, 1938; GOLDRING, CHASIS, RANGES and SMITH, 1940; SMITH, 1941). But it was also shown that the tubular excretion varied with the plasma concentration of the substance in question as long as this latter was low. At rising plasma concentration, however, the tubular excretion became constant and the clearance fell. This "depression of the clearance" was taken to imply that the tubular excretory rate is limited by the inability of the tubular cells to transfer more than a certain amount of the substances per time unit from the peritubular fluid to the preurine. This maximal rate —  $T_m$  — would, then, reveal the amount of functioning tubular tissue available for the excretion in question — tubular excretory mass.

## 3. The reabsorptive function of the tubules

After filtration in the glomeruli the greater part of the filtrate is reabsorbed in the tubules. The tubule's capacity of reabsorbing certain substances is, however, limited, wherefore the tubular apparatus comes to render its regulating effects on the composition of the blood. In other words, the reabsorption would form the "threshold" whereby the body partly regulates the amount of "threshold substan-

ces" in the blood. Although the majority of substances in the blood are such threshold substances, only few of them have been the object of closer investigations in this respect.

In frog and *Necturus* it has experimentally been possible to follow the reabsorption along the tubules through collection and analysis of tubular fluid, thus gathering a conception above all of the localization of the reabsorbing epithelium (see RICHARDS, 1935). Similar investigations have also been done on rabbit (WALKER, BOTT, OLIVER and MACDOWELL, 1941). It appears from these investigations that for instance glucose is reabsorbed exclusively in the proximal tubules, while on the other hand chloride mainly is reabsorbed in the distal part of the tubules.

In mammals our comprehension of the mechanism of reabsorption is mainly founded on clearance tests, showing that e. g. a number of aminoacids are but partly reabsorbed in low plasma concentrations (PIRTS, 1943), while other substances are practically entirely reabsorbed. Examples of the latter are glucose and ascorbic acid, whose reabsorption will here be studied in further detail. First, however, the question of the reabsorption of water will be dealt with briefly.

**The water reabsorption.** Our knowledge of the water reabsorption is as yet minimal. If the values submitted for the filtration are accepted, then 99 per cent of the filtered water is reabsorbed, and since it has been found that creatinine and inulin clearance are independent of the diuresis, then this latter must be regulated by the water reabsorption. In frogs whose tubules had been blocked with phlorizin, WALKER and HUDSON (1937) observed a concentration increase of glucose in the proximal part and therefore assumed a water reabsorption there. Another concentration of preurine has, however, been found in the thin segment of Henle's loop (CRANE, 1927). On other grounds, too, this part of the tubules has been assumed to be the site of the water reabsorption: PETER (1909) and MARSHALL (1934), have pointed out that only those animals where the thin segment is developed are able to produce hypertonic urine. The hypothesis is drawn up that the reabsorption of water is divided into two phases: first, an obligatory process, which is essentially isotonic in nature, and which takes place in the proximal part, and, second, a variable reabsorption, potentially hypertonic, assumed to take place in Henle's loop and to be influenced by the antidiuretic hormone.

**The reabsorption of glucose.** In frogs and *Necturi* the glucose concentration of the glomerular filtrate falls rapidly after the fluid enters the proximal tubules (WHITE and SCHMITT, 1926; WALKER and HUDSON, 1937). Perfusions of various parts of the tubules with

Ringer's solution containing glucose demonstrate that the reabsorption is limited to the proximal segment. In animals poisoned with phlorizin the concentration remains at or above the plasma level throughout the tubules and appears in the ureters in high concentration. Consequently, the glycosuria produced by phlorizin is due to an inhibition of reabsorption.

In dogs POULSSON (1930) demonstrated that during phlorizin poisoning the kidneys concentrate the glucose approximately in the same degree and in close parallelism with creatinine. NI and REHBERG (1930) experimentally showed that the glycosuria occurring at high concentrations of plasma glucose is due, not to a decrease in the reabsorptive rate but to the fact that more glucose is filtered than can be reabsorbed. They pointed to two possible explanations of this limitation. Either the tubule cells are only able to transfer a certain fixed amount from filtrate to peritubular fluid per unit time, or else a maximal difference in concentration between blood and urine at the place for reabsorption may act as a limiting factor. From their experiments they drew the conclusion that the reabsorption increases with the plasma concentration, even when the latter was high, but that the tubule cells were not able to reabsorb glucose at a concentration difference higher than 600 to 700 mgm per ml. They therefore thought it possible that the concentration difference limits the reabsorptive rate. Using dogs, SHANNON and FISHER (1938) determined the reabsorption under continuous intravenous injection of glucose. As long as the plasma concentration remained below the threshold value practically all filtered glucose was reabsorbed, but above this value the reabsorptive rate remained constant, however great the plasma concentration, and therefore the filtered quantity per unit time was increased. To investigate whether the concentration difference according to Ni and Rehberg had any effect on the reabsorption, they raised the plasma glucose concentration very strongly in a decerebrated dog, afterwards lowering the filtration by half with an aortic clamp. Both before, during and after this operation the reabsorption showed the same value. From this they concluded that it was not the diffusion pressure but an actual maximal rate that limits the reabsorption, i. e. the mechanism of transfer behaves as though it were saturated with respect to the solute. Further investigations have substantiated this (GOVAERTS and MULLER, 1938; SHANNON, FARBER and TROAST, 1941).

This maximal reabsorptive rate — glucose  $T_m$  — proved, as did diodrast  $T_m$ , to be fairly constant in one and the same animal. Glucose  $T_m$  was therefore recommended as a dependable means for the quantitative characterization of tubular (proximal) reabsorptive function. GOLDRING, CHASIS, RANGES and SMITH (1940) have also been able to show a certain correlation between diodrast  $T_m$  and glucose  $T_m$  in man (only few cases). — The excretion rate of glucose proved — according to the cited papers — independent of the degree of diuresis.

The further determination of that plasma concentration value at which the glucose excretion sets in is complicated by several factors. First a minimal excretion of the substance is found even in low concentrations of the plasma glucose (HARDING, NICHOLSON and ARCHIBALD, 1936); second, at rising plasma concentrations we will not find a marked increase of the excretion but a rather gradual one above the "threshold value".

For clinical purposes empirical definitions have had to suffice, but investigations on the threshold value for glucose are few and uncertain. FABER and HANSEN (1923) found in non-diabetics that the threshold value could vary within rather wide limits in different individuals but that it was fairly constant in one and the same person. In certain physiological and pathological states the threshold value may fall, however, and in a material of non-diabetic pregnancies CHASE (1932) reported glycosuria in 60 per cent with normal plasma value. A lowered value is considered to explain the glucosuria in so-called renal diabetes.

/ The reabsorption of ascorbic acid. The few investigations which as yet have been done on the renal excretion of ascorbic acid, have shown that this takes place in almost identically the same manner as the glucose excretion.

Even before the discovery of ascorbic acid VAN EEKELEN, EMMERIE, JOSEPHY and WOLFF (1933) had been able to demonstrate the antiscorbutic action of urine from hypervitaminized individuals. HARRIS, RAY and WARD (1933), and JOHNSON and ZILVA (1934), showed that persons living on a diet poor in Vitamin C only excreted slight traces of ascorbic acid in the urine, whereas the excretion of those on a diet rich in Vitamin C was, on the whole, proportional to the intake.

Using Richards technic LEBLOND (1938) was able to show that the capsular fluid in frog contains ascorbic acid in the same concentration as the plasma; and after intravenous injections on guineapigs the substance could with histological technic be demonstrated in large quantities in the proximal part of the tubules, but not in the distal part (GIRAUD and LEBLOND, 1937).

The quantitative conditions in the renal excretion of ascorbic acid in mammals are elucidated by experiments, above all by SHERRY, FRIEDMAN, PALEY, BERKMAN and RALLI (1940) in dog, by RALLI, FRIEDMAN and RUBIN (1938) and by FRIEDMAN, SHERRY and RALLI (1940) in man. They have here followed the same procedure as Shannon and Fisher in the above mentioned investigations with glucose. The ascorbic acid clearance has thus been studied under concentration balance, in various plasma concentrations over as well as under the threshold value.

With low plasma values they found ascorbic acid clearance very low — though not zero — but constant and independent of plasma concentration. When this concentration had exceeded about 1.5 mgm per cent, however, the clearance rose rapidly and approached the inulin clearance. They also showed that this increase of the clearance depends on a limitation in the capacity of the renal tubules to reabsorb ascorbic acid and that this limitation is absolute. Thus, the same conditions should be valid for ascorbic acid as for glucose: the tubules can but reabsorb a certain maximal quantity per unit time — *ascorbic acid*  $T_m$ ; as long as the filtered amount per unit time falls below this, only negligible amounts of ascorbic acid are excreted with the urine, but when the filtered amount exceeds  $T_m$ , the surplus is excreted in the urine.

SHANNON (1938) has shown that the elevation of the plasma glucose to the level of frank glycosuria completely and reversibly blocks the reabsorption of xylose, and he therefore concluded that xylose is reabsorbed by a process which is common with that responsible for the reabsorption of glucose. Since glucose and ascorbic acid resemble one another in chemical structure RALLI, FRIEDMAN and RUBIN (1938) made a further investigation to see if the mechanism for these two substances, too, was common. This was not the case.

The same difficulties as in the case of glucose are encountered in endeavouring to determine the threshold value for ascorbic acid. The critical level of excretion seems to be between 1.5 and 2 mgm per



cent, dependent on the methodics and manner of determining the threshold value (RALLI et al., 1938; FAULKNER and TAYLOR, 1938, and others). — Neither does the excretion of ascorbic acid seem to be influenced by the degree of diuresis, according to the quoted authors, and in published material by FRIEDMAN and co-workers one finds no change in the filtration rate on changes in plasma ascorbic acid concentration.

Some authors suggest the employment of ascorbic acid in the determination of renal function. Thus SENDROY and MILLER (1939), in investigations on the Vitamin C standard in man, found that abnormally slow excretion of administered ascorbic acid does not necessarily indicate a low ascorbic acid content of the body when renal function is low. They also considered themselves to have established that the effect of lowered kidney function on the ascorbic acid clearance runs approximately parallel to other kidney function tests (urea clearance). Likewise, LUDDEN and WRIGHT (1940) call attention to the effect of renal retention of ascorbic acid on saturation tests. More systematic investigations on the applicability of ascorbic acid in renal function tests have not, judging from the available literature, been done.

#### 4. Summary

Our theories concerning the renal function in man are still to a great extent founded on observations on lower mammals. Recent years' clearance investigations on man have, however, to a certain extent verified the renal function theory, already suggested by Cushny — involving an ultrafiltration in the glomeruli, caused by the hydrostatic pressure, and an active reabsorption in the tubules.

Arguments have in the literature been propounded for the following assumptions, of immediate interest for this work:

1. The filtration rate in the glomeruli under physiological conditions, determined by inulin clearance, is remarkably constant.
2. In quantitative investigations the human kidney may be considered as a single nephron.
3. The degree of diuresis does not affect the reabsorption rate for glucose or ascorbic acid.
4. The reabsorption of glucose and ascorbic acid apparently takes place in identically the same manner.

5. Not until above a certain critical plasma concentration value — the threshold value — does any considerable excretion of these substances set in.

6. The reason for this seems to be that the reabsorption rate has a constant absolute upper limit —  $T_m$ . As long as the filtered amount per unit time remains below  $T_m$ , practically all is reabsorbed in the tubules; when the filtered amount exceeds  $T_m$ , the surplus is excreted with the urine.

## II

### The problems

If ascorbic acid is present in the plasma in a concentration of  $P$ , and if the filtration rate — i. e. the amount of fluid filtered in the glomeruli per time unit — is  $F$ , then the filtered quantity of ascorbic acid per unit time is  $PF$ , and if the amount reabsorbed in the tubules and excreted with the urine per unit time is  $T$  and  $S$  respectively, then in general

$$PF - S = T \quad (2)$$

Thus, the reabsorptive rate can be determined if we know the plasma concentration, the filtration rate and the excretion rate.

If, however,  $P$  — being assumed greater than the threshold value in order to secure a maximal reabsorptive rate — and  $S$  are permitted to vary, while  $F$  and  $T$  remain constant according to what has been stated above, the value of these constants can be determined as has been done diagrammatically in fig. 1.  $T_m$  — maximal reabsorptive rate — can be read off directly at the intersection on the y-axis while  $F$  will be equal to  $\text{tg } \alpha$ . Or in other words, if we know two points,  $P_1S_1$  and  $P_2S_2$ , on the curve — then  $F$  and  $T_m$  can be solved according to the equation system

$$\begin{cases} P_1F - S_1 = T_m \\ P_2F - S_2 = T_m \end{cases} \quad (3)$$

In theory then, *if we know the plasma concentration and the excretion rate on two separate occasions above the threshold value, it will be possible to calculate both the glomerular filtration rate ( $F$ ) and the maximal tubular reabsorptive rate ( $T_m$ )*. The possible combinations are shown in fig. 2.

Of the two quantities,  $F$  has for a long time been employed as an applicable measure for one of the functions of the kidney — the glomerular function — while we for the other, the tubulus function, have

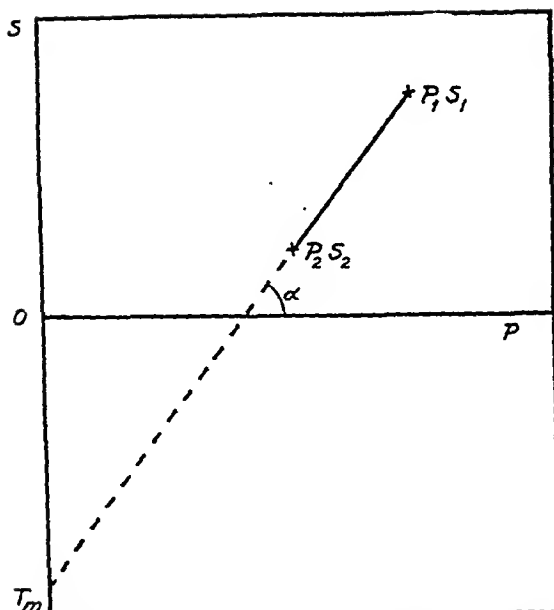


Fig. 1. The theoretical relation between the plasma concentration ( $P$ ) and excretion rate ( $S$ ) for ascorbic acid at high plasma concentrations. The value of  $\text{tg } \alpha$  is determined by the filtration rate, the value of  $T_m$  by the maximal reabsorptive rate. See text.

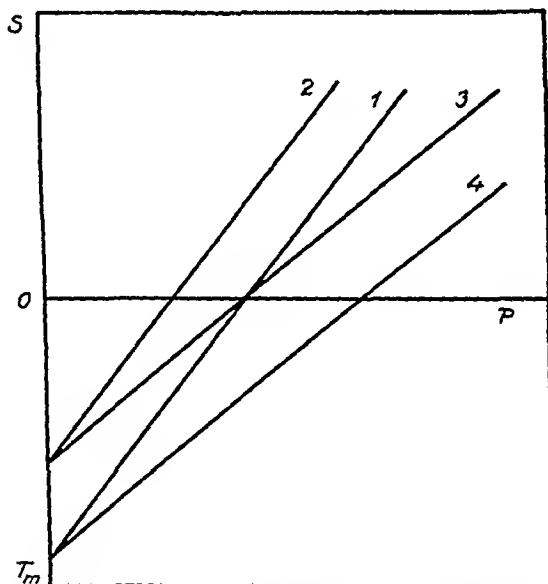


Fig. 2. The inclination and course of the tentative excretion curve for ascorbic acid at high plasma concentrations. 1. Normal case. 2. Lowered reabsorption capacity and normal filtration rate. 3. Lowered reabsorption capacity and lowered filtration rate. 4. Normal reabsorption capacity and lowered filtration rate.

lacked an available gauge. It has, however, already been pointed out (see above) that the requirements are on hand for considering  $T_m$  as a quantitative characterization of tubular (proximal) reabsorptive function. If the above quoted theoretical arguments were realized and verified experimentally, we should obtain a method by which to determine the glomerulus function and a tubulus function *by one and the same examination*.

It has thus been the object of the present study to examine the premises for such a combined renal function test.

In the here published results of the investigations — covering a physiological material only — Section III gives the methods used, after which follow the investigations on the excretion at low, rising plasma concentrations in order to determine the threshold value (Section IV). Section V gives the obtained values from the excretion curve of the maximal reabsorptive rate and filtration rate. Finally certain questions related to the nature of the reabsorptive mechanism are discussed (Section VI).

### III

## Methods

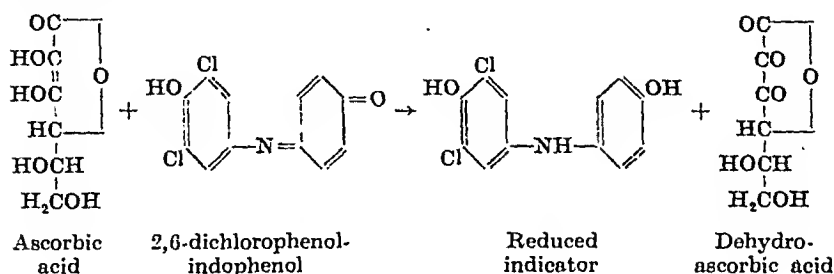
### 1. Statistical methods

The statistical treatment of the material has been done according to the usual formulas as given by FISHER (1930). The method error has been calculated from the differences in two or more determinations on the formulas used by ENGHOFF (1937, p. 109).

### 2. The determination of plasma ascorbic acid

*Chemical data, etc. of ascorbic acid* (SVIRBELY and SZENT-GYÖRGYI, 1932; JOHNSON and ZILVA, 1934; BORSOOK, DAVENPORT, JEFFREYS and WARNER, 1937; KING, 1939). 1-ascorbic acid, whose brutto formula is  $C_6H_8O_6$  (see below), is a monobasic acid and a powerful reducing agent. Its acid properties (acidic dissociation  $pk_1 = 4.17$ ) are said to be due rather to dissociation of an enolic hydrogen than to opening of the lactone ring.

Its oxidation takes place in well demarcated steps, of which the first leads to dehydroascorbic acid — e. g. when ascorbic acid in acid solution is made to reduce dichlorophenol-indophenol:



This oxidation product can be reduced quantitatively to ascorbic acid with, for example, hydrogen sulphide. In aqueous solutions with a pH larger than 5, ascorbic acid is oxidized very readily by the air oxygen in the presence of copper as catalyzer. This reaction is greatly accelerated by exposure to light.

Ascorbic acid is, however, non-autooxidizable within the normal pH range of animal tissues, and it is not changed quantitatively in the bladder (SHERRY

and FRIEDMAN, 1939). — Unlike certain other animals, the primates cannot synthesize ascorbic acid.

In the present investigation ascorbic acid has been administered in the form of *Redoxon Roche forte pro inj.* (100 mgm/ml) or of *Tabl. Redoxon Roche*, containing 50 mgm ascorbic acid.

A number of methods have been worked out for determining ascorbic acid in a biological medium, all based on its power of reduction. The indicators most commonly used have been 2,6-dichlorophenol-indophenol (TILLMANS, HIRSCH and HIRSCH, 1932) and methylene blue (MARTINI and BONSIGNORI, 1934). However, both plasma and urine contain reducing substances in minimal quantities, which disturb the titration — above all those of the R-SH type, cysteine, glutathione and other. Most methods are therefore encumbered with appliances for eliminating the effect of these substances.

After the severe criticism by, among others, DAGULF (1939) and KIRK and WARBURG (1940), directed against the use of methylene blue, it now appears to be abandoned as an indicator. Besides, a method for dichlorophenol has been worked out, which makes use of the fact that disturbing substances usually react slower with the dye than does the ascorbic acid.

The principle of this method, published in 1937 by MEUNIER, and applied to the analysis of plasma by MINDLIN and BUTLER (1938), is to make a certain amount of the sample reduce a surplus of dye solution, and to determine the degree of reduction by means of a photo-electric colorimeter. This eliminates the error of subjective detection of the end-point, and an accurately standardized dye is not needed. The observations of the rate of fading of the dye with time permits the detection and estimation of reducing substances reacting at a slower rate than ascorbic acid. By a suitable choice of the time for reading off it is possible to exclude the disturbing effect of cysteine (MINDLIN and BUTLER, 1938) and of glutathione (BESSEY, 1938). By working in a sufficiently acid medium the influence of other, in the blood not normally occurrent substances, phenols, tannins, etc. is excluded (BESSEY, 1938).

Investigations on the dichlorophenol-reducing influence of plasma under different conditions are numerous. MINDLIN and BUTLER (1938) recovered practically 100 per cent of ascorbic acid, added to plasma. BRYAN, TURNER, HUENEMANN and LOTWIN (1941) were able to show that in healthy human subjects, the level of the fasting plasma ascor-

bic acid is a function of the average daily intake of the vitamin. When using the method in question, one often encounters zero values in test subjects with low vitamin C standard. HJÄRNE (1942) has not less than 34 such cases in his material, and CRANDON, LUND and DILL (1940) communicate that they obtained first decreasing values and later the value zero in repeated examinations of the plasma concentration in their test subject, who had been on a diet free from vitamin C for some time. We have observed 5 cases with reproducible zero values, one of which has been published in another connection (AHLBORG and BRANTE, 1940).

Experiences of the method are favourable throughout and no observations which might be interpreted as caused by disturbing substances have been noted in the investigations in which it has been used (BESSEY, 1938; MINDLIN, 1938; CUSHMAN and BUTLER, 1938; BUTLER and CUSHMAN, 1939; HEINEMANN and HALD, 1940; FRIEDMAN, SHERRY and RALLI, 1940; SHERRY et al., 1940, and others). It should, however, be borne in mind, when placing an equality sign between dichlorophenol-reducing substances and ascorbic acid, that such disturbing substances *may* exist.

*Apparatus:* Photo-electric colorimeter of usual type (see e.g. EVELYN, 1936, or LANGE, 1942) with a Siemens' light spot galvanometer. Here, however, only one selenium sperr cell and a movable tube-holder were used. Sufficiently constant illumination was secured by employing heavy duty storage cells, which were continuously charged from the net. As the acid dye solution showed a maximal absorption at about 520 m $\mu$ , a Wratten filter No. 74 with a maximum at 530 could be used.

*Procedure*<sup>1</sup> (micro-method of MINDLIN and BUTLER, 1938): After hyperemization of the hand in a water bath, about 0.5 ml blood is taken from the fingertip in a heparinized pipette; intense centrifuging for 3 minutes in a narrow tube, after which exactly 0.1 ml plasma is pipetted off and put into an ordinary centrifuge tube. 0.15 ml aq. redest. is added with the same pipette and the protein is precipitated with 0.2 ml 6 per cent metaphosphoric acid; the mixture is shaken and centrifuged strongly for 3 minutes.

Meanwhile 0.8 ml of a buffered dye solution has been pipetted into the colorimeter tube. This solution consists of equal parts of sodium acetate solution, whose pH has been adjusted to 7.0, and dichlorophenol-indophenol solution, whose concentration should vary according to the ascorbic acid concentration expected in the plasma sample. Thus, for concentrations between 0 and 2 mgm per cent the blank should give a reading of about 85 galvanometer scale divi-

<sup>1</sup> Our method differs somewhat from the original method: potassium oxalate has been exchanged for heparin and we have, according to CUSHMAN and BUTLER (1938), not used potassium cyanide.



sions, when distilled water reads 100, whereas the reading should be about 80 when the concentrations exceed 2 mgm per cent. The final reading of the sample will thereby be made within the most sensitive region.

0.3 ml protein-free plasma is pipetted off with great care and introduced into the colorimeter tube, after which the contents are mixed. The galvanometer reading is done exactly 20 seconds after mixing.

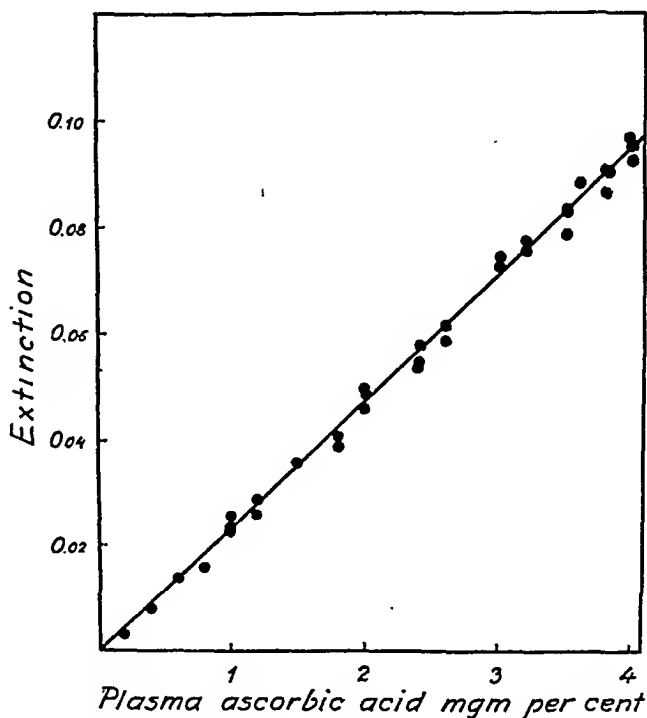


Fig. 3. The extinction at different plasma-ascorbic acid concentrations.  
 $n = 35$ ;  $a_1 = 0.024 \pm 0.0001$ .

The calculation is done according to the formula

$$P = K (\log Gs - \log Gb);$$

where  $P$  is the plasma concentration in mgm per cent,  $Gs$  the galvanometer reading for the sample and  $Gb$  that for the blank. The constant  $K$  has been established according to the extinction curve in fig. 3 by determinations of known plasma-ascorbic acid solutions. For the present apparatus and dilution it will be equal to 42.1.

With a little practice, very satisfactory results can be obtained from the method if the following *precautions* are taken. Haemolysis in the blood sample invariably gives too low values. This can, how-

ever, very easily be avoided by making the hyperemia so intense that the blood runs spontaneously from the lancet wound, and by using heparin instead of oxalate. — The buffered dye solution displays a spontaneous fading, which, though reduced by cold and darkness, cannot be disregarded in experiments of long duration. In the investigations presented here, the strength of the dye solution was, when possible, determined before each plasma analysis. The experiments including concurrent determination of the inulin concentration left no time for this procedure. The fading of the dye, however, proved to be practically linear to time and it was therefore sufficient to determine *Gb* before and after the entire experiment. The value of *Gb* at any given moment could then be read off from a time curve. — Metaphosphoric acid passes readily into orthophosphoric acid, causing a change in *pH*. If the solution is made afresh daily before the experiment and if only the central part of the metaphosphoric acid crystals is used, this trouble is eliminated.

It has been possible in 60 double determinations to reproduce plasma figures within  $\pm 0.04$  mgm per cent. This error is practically the same whether calculated on plasma ascorbic acid values varying between 0.2 to 2.0 or between 2.0 to 4.0 mgm per cent.

### 3. The determination of urine ascorbic acid

When determining the ascorbic acid in the urine, the possible presence of other reducing substances must not be left out of account, and indeed small amounts of such substances have been demonstrated (see e. g. BESSEY, 1939). Various attempts have been made to eliminate their action, precipitating with mercuric and barium acetate etc., but these procedures take a relatively long time and by using them we would have been forced to wait with the urine analyses till after the end of the entire experiment. This would, however, have entailed a loss of ascorbic acid in the samples on standing (WRIGHT, 1936).

EVELYN, MALLOY and ROSEN (1938) have shown that determinations of urine ascorbic acid, too, will present lower values if photo-electric titration is performed on the same principles as hold good for plasma determinations. If a strongly acid medium is used, the ascorbic acid reacts very quickly with dichlorophenol-indophenol,

at the same time as the action of certain of the disturbing substances is obviated. They have also utilized the time factor even more than MINDLIN and BUTLER in their experiments with plasma, and recommend reading off the extinction every 5th second and extrapolation of the resulting curve when the reaction between the ascorbic acid and the dye can be considered over — in practice, at the time zero. Needless to say, this extrapolation will be more exact the earlier the first reading can be made, and the apparatus used by Evelyn has made this possible no more than 5 seconds after mixing. This was not possible for the experimental gear used in the present investigations, however; we had to content ourselves with a first reading 15 seconds after mixing. Evelyn furthermore carried out the extrapolation according to a curved line. As the equation of the line hardly can be calculated, it seems difficult to make such a numerical extrapolation. Instead, the straight connecting-line between the extinction at 30 and at 15 seconds has been taken to the y-axis, and the intersection of these two regarded as the extinction caused by ascorbic acid (see Fig. 4). In this way the values probably become higher than those obtained with the original methods, and it has therefore been necessary to make clear the error involved.

As has already been briefly mentioned, FRIEDMAN, SHERRY and RALLI (1940) — who used the methods of Evelyn, Malloy and Rosen — found in very careful investigations that ascorbic acid is excreted with the urine even when the concentration in the plasma is low, i. e. below the threshold value. They assumed they were dealing with ascorbic acid from the circumstances that there was a general correlation between the plasma concentration and the excretion. The excretion was nevertheless very small, amounting to a maximum of about 0.035 mgm per minute.

In his extrapolation procedure the present author has on 15 experimental subjects carried out 85 determinations of the excretion rate of dichlorophenol-reducing substance at different plasma concentrations under 1.5 mgm per cent, and found in so doing that the mean for this "rest excretion" was  $0.020 \pm 0.0015$  mgm per minute. Even if this is for the most part conditioned by disturbing substances — which is hardly likely in the light of the above described investigation — it is so small as to have no effect whatsoever on the results in the present investigation.

It has naturally been necessary at high concentrations to dilute

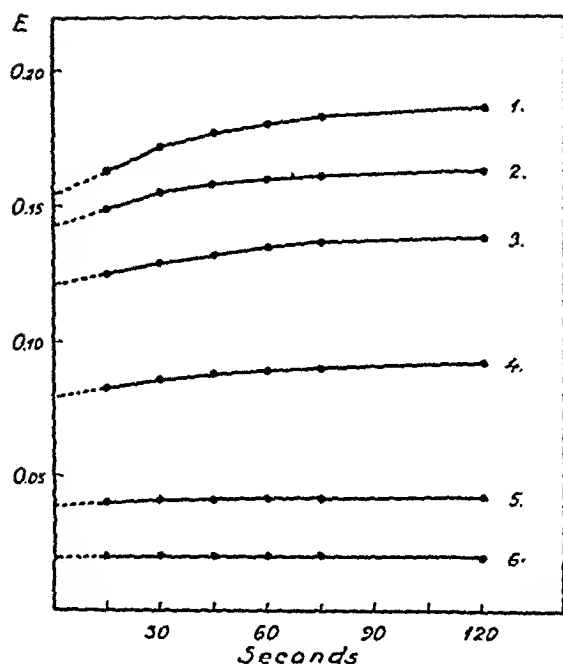


Fig. 4. The extinction at different dilutions of one and the same urine sample. The time in seconds after the mixture of the sample with dichlorophenol-indophenol solution. Regarding the extrapolation to the time 0 ( $E_0$ ), see text.

No.	Dilution urine : water	$E_0$	Ascorbic acid	
			Mgm per cent	Mgm per 100 ml urino
1	Undiluted	0.154	3.78	3.78
2	7 : 1	0.143	3.52	4.00
3	3 : 1	0.121	2.98	3.95
4	1 : 1	0.080	1.97	3.94
5	1 : 3	0.039	0.96	3.84
6	1 : 7	0.020	0.49	3.92

the urine and the effect of the dilution on the extinction has therefore been investigated. Fig. 4 gives the curves from one of these experiments. The top curve is from the native undiluted urine (diuresis about 2 ml per minute), the bottom one after the urine has been diluted with water in the ratio 1:7. As can be seen from the values obtained from the curves there is good agreement between the calculated amounts of excreted substance. Judging from these investigations, then, the degree of the dilution should not affect the results obtained with this method.

*Procedure.* Apparatus as in the plasma ascorbic acid determination.

The samples of urine were taken either with a catheter or, in the majority of the cases, the subjects emptied their bladders voluntarily. With a high diuresis of 5—15 ml per minute the error of the latter method due to possible residual urine has proved minimal, at any rate in physiological material. The urine was immediately acidified with glacial acetic acid so that the sample contained 5 per cent acetic acid. Blank tests were made as in the plasma determination.

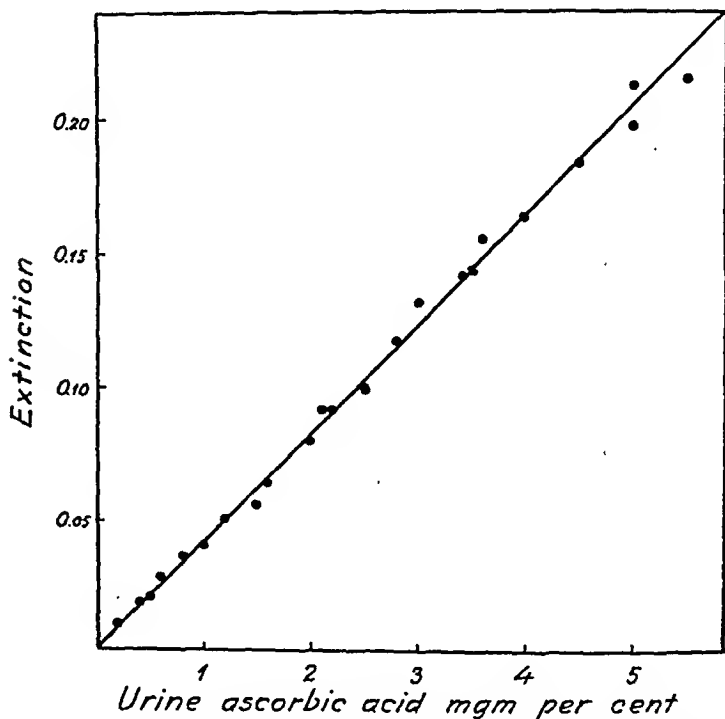


Fig. 5. The extinction at different urine-ascorbic acid concentrations.  
 $n = 24$ ;  $a_1 = 0.041 \pm 0.0003$ .

but with 1 ml acetic acid and 9 ml dichlorophenol-indophenol solution whose concentration should vary according to the ascorbic acid concentration expected (see p. 23). 1 ml of the acidified sample was pipetted into the colorimeter tube, after which 9 ml of the dye solution was added from a pipette whose tip had been removed to make it empty more rapidly. Reading made exactly 15 seconds after mixing, and every subsequent 15 seconds. Since a large quantity of samples are available and the method is very swift, repeated analyses were made, which normally gave accordant values.

The calculation is done according to the same equation as was given for the plasma determinations. With the apparatus and the dilution in question, the constant is 24.6 according to the curve in fig. 5, where the extinction has been determined for known urine ascorbic acid solutions.

#### 4. The determination of inulin clearance

The object of the inulin clearance investigations has partly been, through them to be able to eliminate from the material eventual cases with a definite lowering of the glomerulus function, and partly to procure a measure for the average filtration rate in the material.

As has been seen from the foregoing a microdetermination has been used in the ascorbic acid determination, allowing the blood sample to be taken from the fingertip. The reason for this has obviously been to spare the subjects from — in certain investigations — up to 12 venous punctures in the course of 6 hours. For technical reasons, however, the samples of blood for the ascorbic acid and for the inulin-test had to be taken simultaneously, and we were thus obliged to make a microdetermination for the inulin-analyses also.

The method used here is on all essentials based on that simultaneously introduced by CORCORAN and PAGE (1939) and ALVING, RUBIN and MILLER (1939), and modified by ALVING and MILLER (1940) and HOGEMAN (1943; and in press). As to the applicability of this method for practical use, the reader is referred to the latter author's works.

Inulin, which is a high-molecular polysaccharide, twins into fructose on hydrolysis, and is determined as such according to the method introduced by Alving and collaborator. Fructose reacts to diphenylamine with a blue color and this reaction is specific to inulin in plasma and urine if the glucose (and fructose) is broken down with yeast before the reaction. The reaction can be determined quantitatively by means of a colorimeter.

Among the modifications that of Hogeman will be described, as it has been the basis of our methodics, and we have had the opportunity of making parallel investigations of a number of his cases.

His procedure is approximately this: After the taking of blood samples for blank analysis, 50 ml of *Inulin Astra* (10 per cent) are injected intravenously. The patient is made to empty his bladder one hour after injection, and it is then emptied at intervals of 20–30 minutes during the next 90 minutes. A few minutes before the middle of each period — correction for renal dead space (see below) — blood samples are taken by venous puncture. After all the samples have been taken the glucose in the plasma samples is fermented with or-

dinary washed yeast, after which the protein is precipitated with zinc sulphate. The protein-free plasma is brought together with diphenylamine solution in specially constructed hydrolysis glass ampullae, which are sealed and placed in a boiling water bath. After the samples have been hydrolyzed for one hour, the extinction is read off in a Pulfrich photometer. The urine samples are treated in the same way, but, unless glucosuria is present, are not fermented.

Using this method Hogeman finds a plasma inulin concentration of between 11 and 13 after one hour, and after a further hour between 6 and 8 mgm per cent. In fermented blank samples from the plasma there is a blue coloration corresponding to 0.3—0.7 mgm per cent inulin, in unfermented corresponding to 6—10. The mean for the inulin clearance appears from the tables, page 9, and the method error, calculated from differences in three periods,  $\pm 6$  per cent.

Our methodics differ from that of Hogeman inasmuch as we for one thing use smaller amounts of plasma, and for another do not ferment the samples. A plasma determination is instead done before the injection of inulin, and this blank value is later subtracted from each calculated plasma value. In our method there thus arises an error, brought about by the changes in the glucose concentration during the experiment. This error, however, judging from investigations with the method, seems to be relatively small, but it naturally limits the employment of the method to healthy subjects only.

*Procedure.* Exactly 0.2 ml plasma is taken from the heparinized blood sample, and diluted with 0.3 ml aq. redest. 0.2 ml 10 per cent zinc sulphate and 0.2 ml  $n/2$  NaOH is then added. Violent shaking, and centrifuging for 15 minutes. Exactly 0.4 ml of proteinfree plasma is pipetted off and to this is added 4 ml of the diphenylamine reagent. This has been prepared as follows:

Stock solution I: 1 part concentrated hydrochloric acid  
2 parts redistilled ethyl alcohol

Stock solution II: 1 gram diphenylamine  
12.5 ml redistilled ethyl alcohol

The reagent used consists of 50 ml of solution I and 3.35 ml of solution II. The reagent was made afresh before each experiment.

2 ml of plasma-diphenylamine solution is put into an ordinary drug ampulla, which is sealed. The ampulla is then placed in a boiling water bath, where the inulin is left to hydrolyze for one hour. The urine samples receive a similar treatment after suitable dilution. The extinction is then read off in the usual way in the photoelectric colorimeter. An extinction curve, calculated from known inulin solutions in the customary manner, gives with the filter used — Wratten filter No. 70 — a straight line through origo (cf. Hogeman, cit. above).

With this method we have done 49 clearance determinations on 15 anamnesically sound subjects (see below). During the proceedings it appeared that the plasma concentration at different intervals of time after the injection correlated with corresponding values in Hogeman's material, and, further, that the plasma blanks before the injection of inulin gave a blue coloration due to glucose corresponding to between 5 and 9 mgm per cent inulin. The calculated mean for the whole material ( $n = 49$ ) amounted to  $123 \pm 2$  ml per minute and  $1.73 \text{ m}^2$  body surface<sup>1</sup>, and the total error calculated from the differences in the various periods, was about  $\pm 11$  per cent — or nearly twice as large as in Hogeman's material. Our values for the various periods of 30 minutes are grouped as follows:

1. period  $126 \pm 4$  ml per minute
2. period  $117 \pm 3$  »
3. period  $122 \pm 4$  »

There is no significant difference between the values of the first and second periods.

The error in our methodics, large in comparison with that of Hogeman, is with all certainty due to deviations in the glucose concentration from period to period, rather than to errors in analysis. The method, however, has proved serviceable for investigations on healthy material. For work with clinical material, however, a method with fermentation of the plasma glucose would seem to be a necessity.

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<sup>1</sup> The clearance varies somewhat with the body surface, which, according to MacKAY (1932) can be used as a relative gauge of the size of renal parenchyma. The correction was here done according to a line chart published by PETERS and VAN SLYKE (1931), and giving a correction factor for each body surface.



#### IV

### The excretion of ascorbic acid at rising plasma concentrations

Above it was pointed out that there were good foundations for the assumption that the renal filtration rate in man under physiological conditions may be considered constant. In addition, a review was given of experimental investigations, speaking in favour of the fact that the reabsorption rate for ascorbic acid in the tubules has a certain maximal limit, which is not exceeded, and that this capacity of reabsorption practically speaking always is entirely utilized, given a sufficient supply of ascorbic acid. The consequence of this is, however, that the excretion curve of the substance in question, e. g. the excretion rate at differing plasma concentrations, should have this appearance: at a certain plasma concentration value the excretion should set in and the excretion rate above this threshold value be directly proportional to the amount of filtered substance, i. e. to the plasma concentration (Fig. 6).

The object of the investigation has been experimentally to determine the course of the excretion curve at maximal reabsorption rate, thus, according to the hypothesis, above the threshold value. The plasma concentration in our experiments should accordingly not fall below this value. Our first task will thus be to investigate the extent and variability of this value, with the method indicated.

According to the hypothesis granted, the excretion curve should have the same course whether the investigations are carried out with continuous intravenous injection of ascorbic acid and after the plasma concentration has been constant for some time — *the excretion curve at concentration balance* — or with *rising or falling plasma concentrations*. In other words, the threshold value should be definite and have the same value, whichever the technic used.

It is, however, difficult to determine this value under concentration balance, as such a technic demands intravenous drip. It is also very time-consuming to follow the excretion after an initial intravenous injection all the way down to that plasma concentration value, at which the excretion practically speaking can be considered to cease, owing to the fact that the plasma concentration in the proxi-

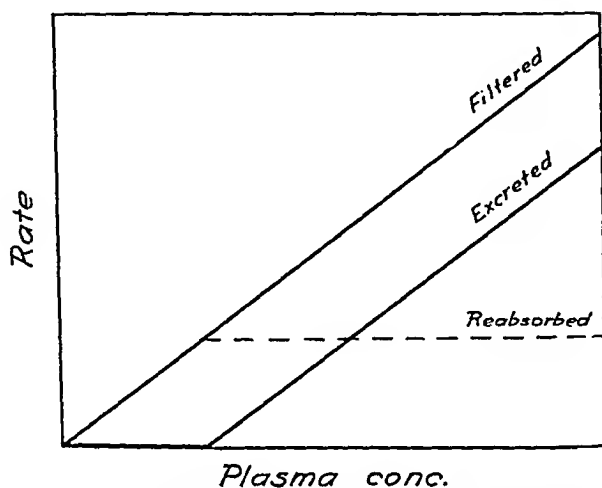


Fig. 6. Theoretically calculated amount ascorbic acid filtered, excreted and reabsorbed per time unit at different plasma concentrations.

mity of the threshold value falls very slowly. In rising plasma concentrations, on the other side, it is, by varying the load, possible to direct the excretion curve. This technic has therefore been employed in determining the threshold value.

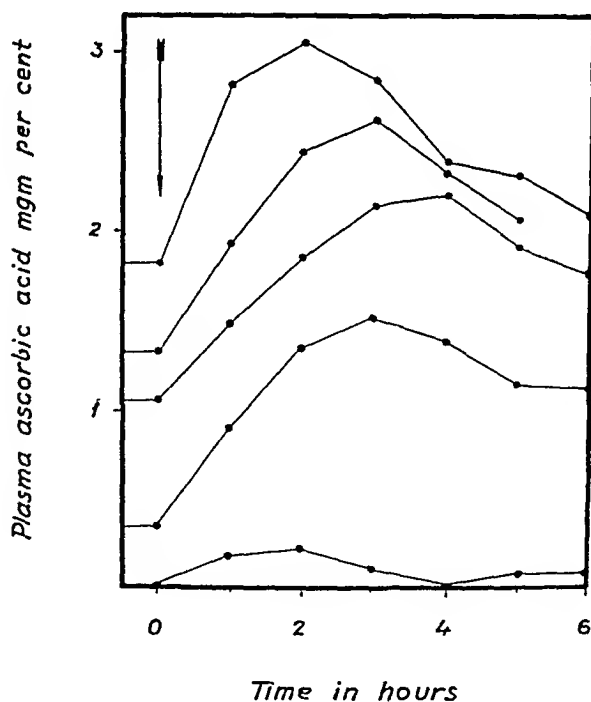
### 1. The loading curve

Normally and fasting the concentration of ascorbic acid in plasma as a matter of fact hardly varies during 4—5 hours, as is shown in table I. After peroral administration of 10 mgm per kilo body weight the concentration rises quite rapidly and reaches its maximum after 2—3 hours, later to fall and remain practically speaking constant again after about 6 hours (Fig. 7). In high values before the loading, a relatively pronounced fall in the concentration takes place, when maximum primarily is reached. It is from earlier investigations evident that this is conditioned by the renal excretion.

Tab. I.

Plasma ascorbic acid in 5 fasting individuals during 2 to 5 hours.

Hours	Plasma ascorbic acid mgm per cent				
	No. 1	No. 2	No. 3	No. 4	No. 5
0	0.13	0.72	1.06	1.21	1.58
1/2			0.98		1.59
1	0.11	0.68	1.03	1.32	1.58
1 1/2			1.06	1.28	1.56
2	0.09	0.65	1.01		1.60
3		0.70		1.30	
4		0.68		1.25	
5	0.11	0.68		1.14	

Fig. 7. The loading curves after administration of 10 mgm ascorbic acid per kilo body weight ( $\downarrow$ ) at various fasting values.

## 2. The excretion curve

On comparative investigations between the plasma concentration and the excretion rate on changes in the plasma concentration, one should on principle demand, *firstly*, that the excretion rate is determined at the exact time at which the plasma sample is taken, and, *secondly*, that the time lag, arising in "renal dead space" i. e. the time taken by the urine to pass through kidney and ureter to the bladder, is known. The first requirement can not be fulfilled, as the urine has to be collected through a relatively lengthy period of time. If, however, attention is given to having the change in plasma concentration take place uniformly, then the mean excretion per minute during a certain period of time may be considered to correspond to the concentration at the middle of the period. The concentration may in turn either be determined at exactly this time, or calculated from the concentration values at the beginning and the end of the period.

The time lag in renal dead space naturally varies with the diuresis, and diminishes in importance in rising diuresis. As will subsequently be shown in another connection, it should in the here occurrent diuresis not exceed 5 minutes, and as the urine is gathered under periods of 30 minutes, it can in these experiments be entirely disregarded.

**Experimental technic.** The plasma concentration has thus been determined every 30 minutes under fractionated peroral loading of ascorbic acid; during the intervening periods the urine has been collected and analysed for the content of ascorbic acid. The procedure is exemplified in fig. 8. In calculating the excretion curve the mean concentration value of 2 successive plasma samples has been compared with the mean excretion per minute during the intervening period of time.

The material has consisted of 24 healthy students, and the investigations have been commenced in the morning, fasting. In the beginning a catheter was used in order to empty the bladder as completely as possible, but it soon was seen that the trial subjects at the diuresis in question ( $> 5$  ml/min.) had no difficulty in emptying their bladders completely without catheter.

**Results.** The investigation revealed that the excretion at plasma concentration values under about 1.7 mgm per cent is minimal, and

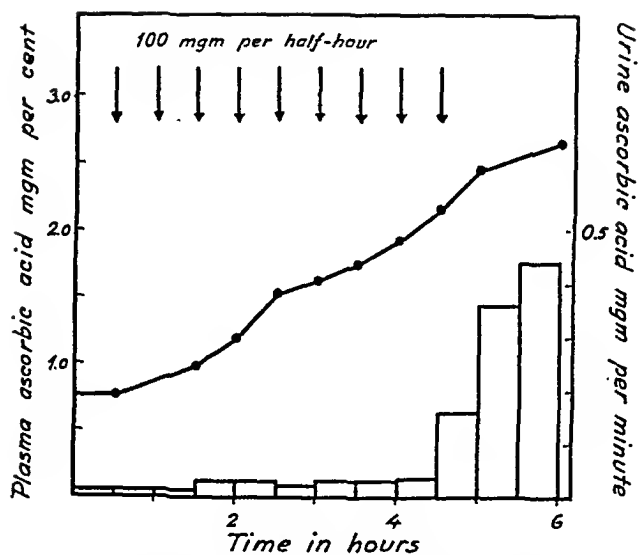


Fig. 8. Plasma concentration (points) and excretion (staples) of ascorbic acid at fractionated peroral loading.

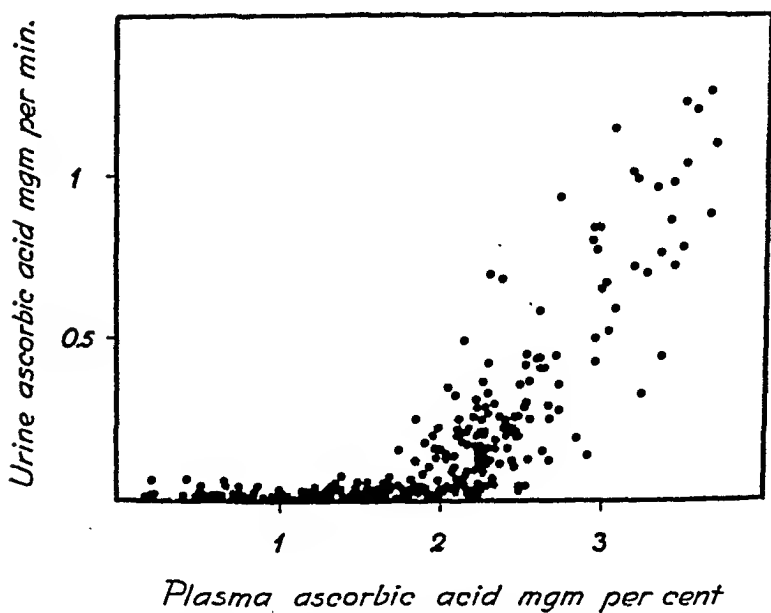


Fig. 9. The excretion rate of ascorbic acid at rising plasma concentrations. The entire material.

in reality at the most reaches but 0.07 mgm per minute, the "rest-excretion". At about 2.0 mgm per cent the excretion rises rather suddenly, but it will be seen (Fig. 9) that the variations are relatively large.

On further analysis of the individual test subject's excretion curves one finds in the first place rather large dissimilarities in appearance. The excretion curve in fig. 10 approaches in shape that which was

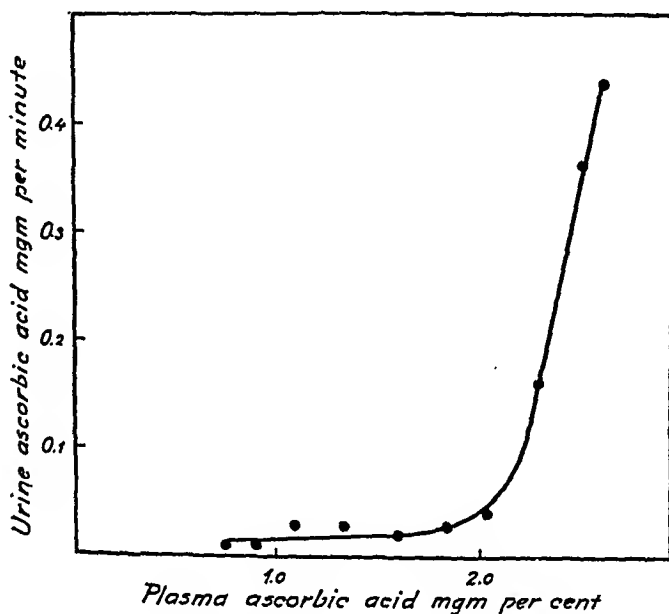


Fig. 10. Excretion curve for experiment, fig. 8.

theoretically computed, as it at a certain value suddenly curves and then remains linear, while on the other side the right curve in fig. 11 shows a considerably more uniform curvature and do not become linear at the plasma concentrations in question under the experiment. In addition, one finds, as is shown in fig. 11, a certain reproducibility of the excretion curve in one and the same individual at different times.

**Discussion.** The investigation of the excretion curve at rising plasma concentrations thus reveals that ascorbic acid is a threshold substance with a critical excretion value of about 2 mgm per cent. It also shows that the excretion curve is reproducible in one and the

same subject on different occasions, but that large individual variations occur.

There is in low plasma concentrations also a minimal excretion, not exceeding 0.1 mgm per minute. This is the first disparity between the theoretically computed curve and that experimentally found (cf. figs. 6 and 9) but may be explained by disturbing substances.

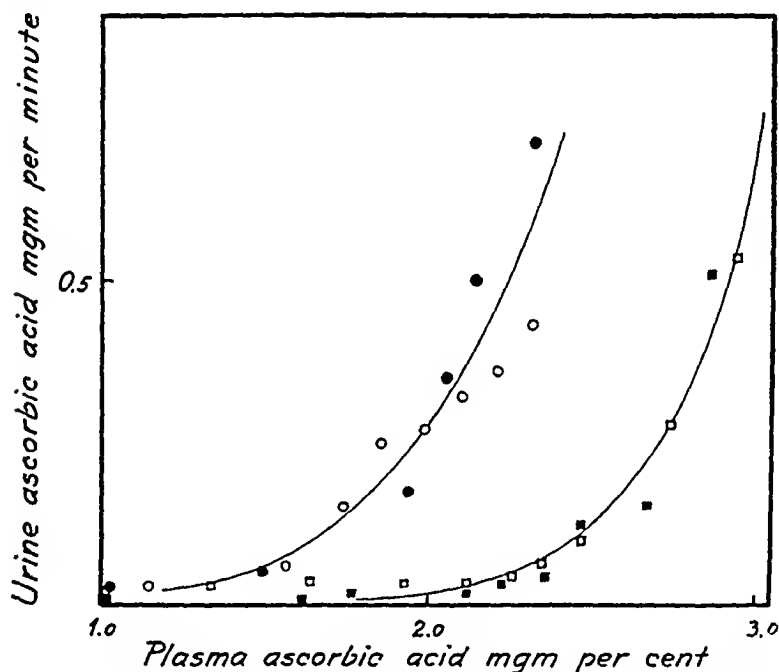


Fig. 11. The excretion curve in two individuals (circles, resp. squares) on two separate occasions (open, resp. filled out). Time lapse between experiments approx. two months.

Another difference lies in the circumstance that in the experimental curve the excretion rate at higher plasma concentrations does not immediately rise linearly, but rather gradually. In fig. 10, e. g., an augmentation of the excretion rate begins already at a plasma concentration value of below 2 mgm per cent and the curve becomes linear at a plasma value of just above this. In fig. 11 (right curve), at a plasma concentration of above 2.5 mgm per cent no definite rectilinearity is as yet seen.

Thus, however, disappears our possibility of determining an exact threshold value and it would seem more adequate to speak of

a *threshold zone*, which begins at that plasma concentration value at which the "rest excretion" passes over to an increased excretion, ending where the excretion curve becomes linear.

The presence of such a threshold zone can, however, not be explained exclusively by the assumption of a constant filtration rate and an absolute reabsorptive maximum. One or several other factors must cooperate in order to give the excretion curve the shape experimentally found.

Before proceeding to seek an explanation to this difference, we shall, however, point to the fact that similar conditions are observed also at concentration balance (RALLI et al., 1938; FRIEDMAN et al., 1940). The differences can thus not entirely be explained as dependent of the excretion being determined at rising plasma concentrations.

SHANNON (1942) has for analogous conditions in glucose excretion given the following acceptable explanation: If the reabsorptive capacity *in the individual nephrons* is considered constant and equal, while the filtration rate is assumed to differ in different glomeruli, then at rising plasma concentrations, the reabsorptive mechanism in those nephrons in which the filtration rate is greatest will first be saturated and thus excrete the substance. At furthermore rising plasma concentrations more and more nephrons will excrete the substance, this fact being reflected in an augmentation of the excretion rate and thus a curvature of the excretion curve at rising plasma concentrations. Not until the plasma concentration is so high that the maximal reabsorptive rate is reached in all the nephrons, will the excretion curve show a straight course.

According to this theory the excretion curve evidently should reveal such a curved course whether it is determined under concentration balance or rising or falling plasma concentrations. And the less divergency there is in the relation *filtration rate / reabsorptive capacity* in the different nephrons, the more the curve will resemble that theoretically computed. The disparity in the appearance of the curves in figs. 10 and 11 should thus be due to the fact that for the test subject in fig. 10 the above mentioned divergence is less than for the subject in fig. 11 (right curve).

The most important consequence of this assumption is, however, that *a beginning augmentation of the excretion does not necessarily imply that the reabsorptive maximum is reached; instead this is attained*



at a higher plasma concentration value, or, where the excretion curve becomes linear. If it thus comes to determining the reabsorptive rate with the outlined method we must ascertain that the excretion curve actually has a linear course and not rest content with establishing the fact that the excretion is determined above the plasma concentration value, the "threshold value" at which the excretion rate shows an augmentation.

### 3. The excretion at sudden rising of the plasma concentration

It has been considered of interest to study the excretion at sudden rising of the plasma concentration from values below to values above the threshold zone. The object of this has primarily been to form a conception of the rate at which the excretory mechanism goes into function.

**Experimental technic.** As experiment subjects were chosen individuals with known threshold zone and low plasma concentration value ( $<0.5$  mgm per cent). The diuresis was as usual raised to above 5 ml per minute by peroral water administration, and thereafter, through a meticulously adjusted water loading, kept as uniform as possible throughout the experiment. After determination of the rest excretion of ascorbic acid under a number of 5 minutes periods, the ascorbic acid was intravenously injected in a dose of such magnitude that the concentration in plasma was secured above 3 mgm per cent during the rest of the experiment. The injection was done very swiftly and exactly 5 minutes after its termination the bladder was emptied, and then again every 5 minutes. The urine samples were then analysed for ascorbic acid in the usual manner.

After the lapse of some time, when the plasma concentration had fallen to about 1 mgm per cent, the experiment was repeated and finally a similar experiment was carried out, after having raised the plasma concentration to such a degree that it before the injection lies securely above 3 mgm per cent.

**Results.** An experiment of this type is illustrated in tab. II and fig. 12. With an initial plasma concentration of but 0.45 mgm per cent the *maximum* excretion does not arrive until the 5th 5 minutes period

Tab. II.

Tab. II. Effect of intravenous injection of ascorbic acid on excretion at different initial values of plasma ascorbic acid. Cf. fig. 12.

Experiment No.	Time in minutes	Initial plasma ascorbic acid mgm per cent	Diuresis ml per 5 min.	Urino ascorbic acid	
				Mgm per cent	Mgm excreted per 5 min.
I	0	0.46			
	5		18	0.0	0.1
	10		25	0.5	0.1
	15		31	0.4	0.1
	20		26	0.4	0.1
	25	Intravenous injection of 750 mgm ascorbic acid. Bladder emptied.			
	30		29	2.3	0.6
	35		31	7.1	2.2
	40		35	12.8	4.5
	45		34	21.2	7.2
	50		43	22.5	9.6
	55		32	21.0	6.9
	60		36	16.2	5.8
II	0	1.12			
	5		9	3.7	0.3
	10		10	3.4	0.3
	15		16	1.9	0.3
	20		22	1.7	0.3
	25	Intravenous injection of 500 mgm ascorbic acid. Bladder emptied.			
	30		27	9.7	2.6
	35		27	32.2	8.6
	40		29	44.0	12.7
	45		27	45.1	12.1
	50		22	54.1	12.0
	55		16	64.3	10.4
III	0	> 3.0			
	5		9	147.0	13.2
	10		18	46.1	8.3
	15		15	38.8	5.8
	20		24	15.8	3.8
	25	Intravenous injection of 500 mgm ascorbic acid. Bladder emptied.			
	30		33	75.8	24.5
	35		35	50.2	17.3
	40		24	50.0	12.0
	45		33	31.8	10.5

(experiment I), with an initial plasma concentration of 1.12 in the 3d period (exp. II) and with an initial plasma concentration above 3 mgm per cent (exp. III) already in the first 5 minutes period.

**Discussion.** It has been demonstrated that after an intravenous injection of ascorbid acid, of such a magnitude that the threshold

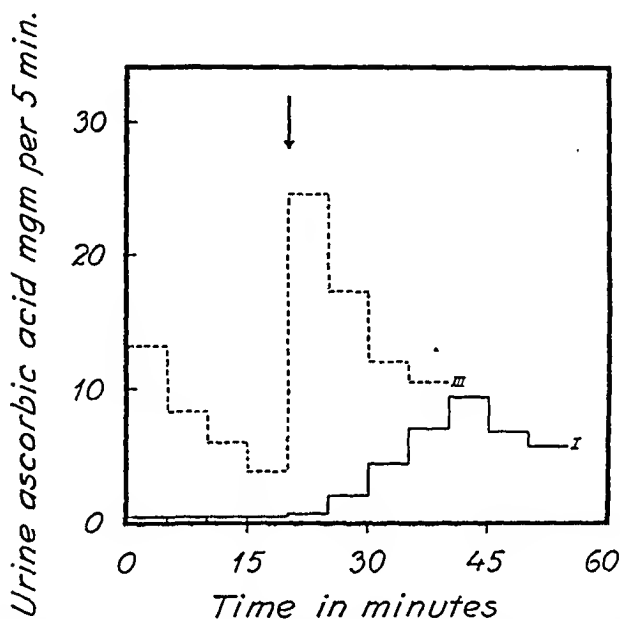


Fig. 12. Excretion rate before and after intravenous injection of ascorbic acid (↓) at initially low (I) and initially high (III) plasma concentrations. See tab. II.

zone is exceeded, the maximum of excretion is attained the earlier, the higher the plasma concentration has been before the injection.

Among conceivable explanations to this retardation of the maximum of excretion in experiments with low initial plasma concentration values the following may be discussed:

A. The maximum of concentration in the blood supplied to the glomeruli is not reached until after the 5th 5 minutes period, or simultaneously with the maximum of excretion.

There is no reason to suspect that the composition of the blood in the renal arteries differs quantitatively from that of the arterics of the finger. It appears, however, from table III that also at very low initial values the concentration of ascorbic acid in plasma from the



13 there was no variation of significance in inulin clearance in different concentrations of plasma ascorbic acid.

D. A gradual fall of the reabsorptive rate after the injection. In figs. 14 and 15 the course of events after the injection (at the time zero) has been reconstructed as regards the amount of filtered, reabsorbed and excreted substance per time unit in experiments I and III. The curves are drawn schematically throughout and no consi-

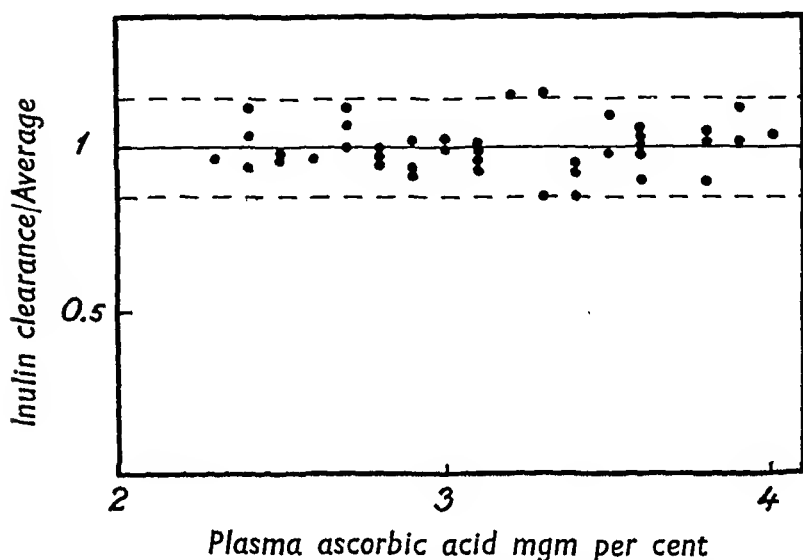


Fig. 13. Inulin clearance at various plasma concentrations of ascorbic acid. The dotted lines limit the area  $\pm 2\sigma$ .

deration to eventual time-displacements after the injection has been taken.

As regards experiment III (fig. 15), it will be seen that the course of the filtration and the excretion curves does not contradict the assumption of a constant reabsorptive rate. Experiment I, however, (fig. 14) can not merely be explained by such an assumption. If nevertheless we stick to the idea of a reabsorption mechanism with constant maximal capacity ( $b$  in fig. 14), we must imagine another reabsorption ( $a$ ) being superimposed on the first one.

Have we any means of explaining the arisal of such a superimposed reabsorption? As mentioned above only a portion of the tubule cells are told off and capable of transferring ascorbic acid from the

preurine and back to the circulation. These cells are thus at the service of the entire organism. It is, however, reasonable to assume that all the tubule cells in some way or other seize a portion of the ascorbic acid in the tubules without passing it on. That being the case, this "private" reabsorption would probably follow the laws valid for the absorption of ascorbic acid in the other cells of the body.

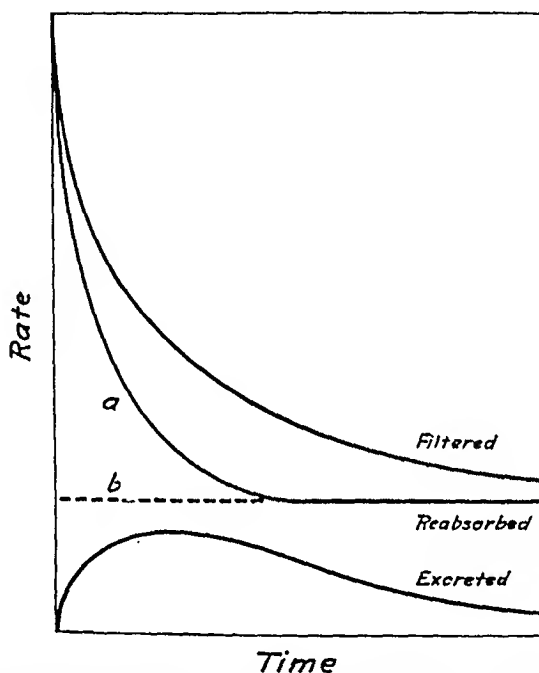


Fig. 14. Amount ascorbic acid filtered, reabsorbed and excreted per time unit after intravenous injection of ascorbic acid at initially low plasma concentrations. Further explanation see text.

As regards the absorption of ascorbic acid in the corpuscles we have the support of rather extensive investigations. HEINEMANN (1938 and 1941) and HEINEMANN and HALD (1940) have been able to show in investigations into the distribution of ascorbic acid between red cells and serum of human blood that it admittedly penetrates to the cells, but that this passage is irreversible and is stopped at low temperatures. This passage therefore appears to be associated with some metabolic activity, and the substance seems to enter into an irreversible union with some intracellular substance or substances. When determining the distribution ratio *serum / cells* at

rising concentrations, they found it to be smaller than unity below about 3.5 mgm per cent, but greater than unity above this concentration. In high concentrations, however, the method error becomes great, so that they do not venture to make any definite pronouncement as to whether there is a maximal load which cells will assume.

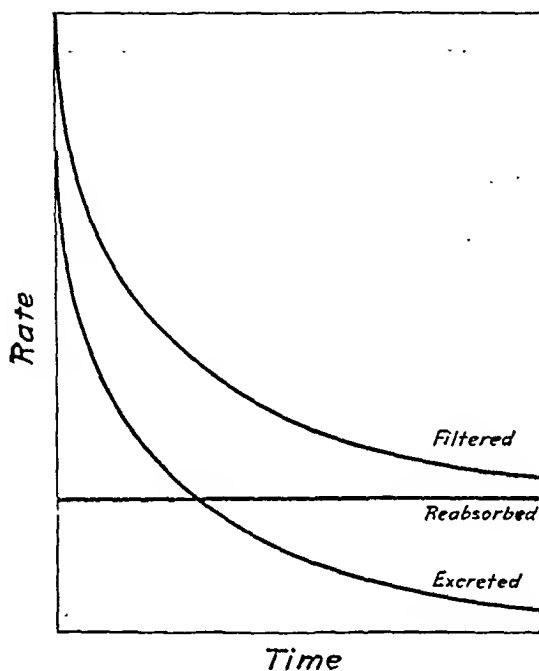


Fig. 15. Amount ascorbic acid filtered, reabsorbed and excreted per time unit after intravenous injection of ascorbic acid at initially high plasma concentrations. Further explanation see text.

The general trend of the observations does suggest, however, that the concentration in cells approaches a limit.

We have experimentally no possibility of similarly investigating the distribution between preurine and tubule cells, but if the same laws hold good for this distribution also, then the consequence should be that at rising concentrations a certain amount is stored in the tubules over and above that reabsorbed by the "public cells". The reabsorption rate should thus be augmented at rising concentrations in the preurine, while one in falling concentrations only should find the ordinary reabsorption and no superimposed reabsorption or tubular "excretion".

The consequence of this would in turn be, that the threshold zone should lie higher at rising than at falling plasma concentrations. That this is the case for ascorbic acid is apparent from the experiment illustrated in fig. 16. That it holds good for glucose has long been acknowledged (HANSEN, 1923; HATLEHOL, 1924; SAKAGUCHI et al., 1924; and others) and has by PETERS and VAN SLYKE 1931, *inter alia*,

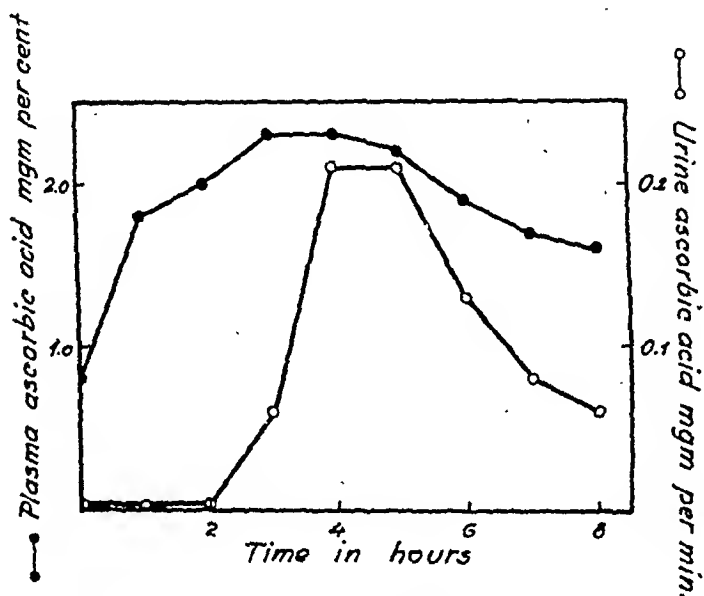


Fig. 16. The excretion at rising and falling plasma concentrations of ascorbic acid. At rising concentrations the excretion sets in at approx. 2 mgm per cent, at falling it still continues at approx. 1.6 mgm per cent.

been interpreted as caused by the kidney's storage of the substance in question.

The displacement of the excretion maximum in the experiment in fig. 12 may apparently until further be explained acceptably as conditioned by a relatively greater reabsorption rate at initially low plasma concentrations (experiment I) than at initially high plasma concentrations (experiment III). This contribution to the reabsorptive rate in the former case would in turn be conditioned by a "private" reabsorption, designed to supply the kidney itself with ascorbic acid.



#### 4. Conclusions

The experimentally determined excretion curve for ascorbic acid thus deviates from the theoretical curve, partly owing to the presence of a threshold zone, and partly because of differences at rising and falling plasma concentrations. None of these deviations, however, necessarily exclude that the filtration rate is constant nor that there is a maximal reabsorptive capacity, but may be explained by divergencies in the relation *filtration rate / reabsorptive capacity* of the individual nephrons, and the kidney's power apart from the usual reabsorption to absorb ascorbic acid without rendering it to the circulation at rising plasma concentrations, respectively.

For determination of ascorbic acid  $T_m$  from the excretion curve the influence of these factors must, however, be eliminated in some way or other. As has been mentioned above there is a possibility that the tubule cells do not render the stored ascorbic acid to the urine at falling plasma concentrations, and that therefore by such a methodic the latter of the disturbing factors may be eliminated.  $T_m$  should accordingly be determined at falling plasma concentrations. To eliminate the former factor it is necessary to work with plasma concentrations above at least 2 mgm per cent and to ascertain that the excretion curve actually has a linear course.

#### 5. Summary

1. The excretion rate of ascorbic acid at rising plasma concentrations between 0 and approximately 1.7 mgm per cent is very low and amounts at the most to 0.07 mgm per minute.

2. Between approximately 1.7 and 2.5 mgm per cent the excretion curve rises suddenly (the threshold zone), subsequently becoming straight. The excretion curves, however, show large individual variations.

3. The threshold zone lies higher at rising than at falling plasma concentrations.

4. The excretion curve is to some extent reproducible in one and the same individual.

5. The established characteristics of the excretion curve may be explained by the assumption of a constant filtration rate and an abso-

lute limited reabsorptive capacity, also assuming that the relation *filtration rate / reabsorptive capacity* is different for different nephrons and that the kidney itself at rising plasma concentrations stores ascorbic acid.

6. For determination of the maximal reabsorptive capacity according to the hypothesis the excretion curve at high falling plasma concentrations appears to be expedient.

## The determination of maximal reabsorptive rate and of filtration rate

A working hypothesis was above suggested according to which it should be feasible to calculate the maximal reabsorptive rate —  $T_m$  — for ascorbic acid, if the plasma concentration —  $P$  — and, simultaneously, the excretion rate for the substance in question —  $S$  — were determined at various values of  $P$ . In addition, a value for the filtration rate in the glomeruli —  $F$  — would at the same time be obtained.

On the basis of the argumentation in the foregoing section experiments have been done at high falling concentrations after an initial dose of ascorbic acid to investigate whether the thus obtained excretion curve is straight, and to determine the equation of this curve.

### 1. Experimental technic

As has been pointed out above the threshold zone lies on an average at about 2 mgm per cent at rising plasma concentrations, with a somewhat higher upper limit. At falling plasma concentrations, on the other hand, the threshold zone should be somewhat lower, and in seeking for the upper limit for the threshold zone in such cases the plasma concentration value immediately above 2 mgm per cent should be the most appropriate. We have therefore investigated the excretion curve within the zone 4 to 2 mgm per cent.

As in the case of several other substances the plasma concentration curve for ascorbic acid after an intravenous injection first shows an immediate rise and then a rather sudden fall, probably conditioned by the distribution to the intra- and intercellular fluid and by the renal excretion. After about 45 minutes the curve becomes somewhat

smoother and we then have optimal conditions for comparative investigations between the plasma concentration and the excretion rate.

Empirically it has been found that if the plasma concentration primarily is raised by peroral loading to about 2 mgm per cent, after which 1 gram of ascorbic acid is injected intravenously, the plasma concentration one hour after injection will lie at about 4 mgm per cent, later falling to about 2 mgm per cent within the next 3 to 4 hours. The determinations should accordingly be done during this period of time.

For these determinations a somewhat different technic has been used than in the experiments at rising plasma concentrations. In order to obtain as accurate values as possible the plasma concentrations have thus been determined in the middle of the excretion period and certain considerations have been taken in regards to renal dead space. In the foregoing part it was pointed out that the dead space of each kidney probably in no normal case exceeds 15 ml. With a diuresis of above 5 ml per minute the time displacement due to renal dead space should accordingly not surpass 3 minutes, and the blood samples are therefore in the present tests taken 3 minutes before the middle of the period.

It was from the beginning our intention to determine the inulin clearance for each period simultaneously with the ascorbic acid investigation. It proved, however, impossible with moderate doses to keep up the concentration of inulin in plasma long enough to obtain reliable values for clearance 3 to 4 hours after injection. In experiments covering more than 2 hours the inulin clearance has accordingly been determined only during the first 2 hours.

As test subjects 17 male students without history or present evidence of renal pathology were employed. Objective tests of their renal condition consisted of albumin tests, investigation of sediment, blood pressure, etc., and in the majority of the cases of inulin clearance. As previously mentioned an inulin clearance value of about 120 ml per minute is in the literature quite unanimously considered as normal, and since we from our original material have eliminated 3 test subjects, the one with a value of above 180 ml per minute, the others with values below 75 ml per minute; the values of the remaining vary but within 105 and 148, which we consider normal (see HOGEMAN, 1943). In 4 of the experiments the inulin deter-

mination was unsuccessful (Exp. No. 200, 203, 213 and 216). For the subject in exp. 200 the inulin clearance was determined some days before the experiment and was found to lie within the above mentioned variation.

The day prior to the experiment the test subject was given so much ascorbic acid — 2 gm *per os* — that the excretion rate amounted to more than 0.1 mgm per minute before the beginning of the experiments. These have begun early in the morning, the test subject fasting and after having rested one hour after arrival to the laboratory. During the experiments he has only risen for having samples taken.

#### Tab. IV.

Experiment No. 214.

Name: N. D. W. Age: 24. Length: 187 cm. Weight: 72 kg.

Correction factor: 0.86. At 10<sup>12</sup> injection of 5 gr inulin and 1 gr ascorbic acid i. v.

Period No.	Time	Urine ml	Ascorbic acid		Inulin		
			Plasma mgm per cent	Urine mgm per min.	Plasma mgm per cent	Urine mgm per min.	Clearance ml per min.
1	11 <sup>00</sup>	Bladder emptied	4.41	3.91	12.0	17.2	143
	11 <sup>12</sup>						
	11 <sup>30</sup>	370					
2	11 <sup>42</sup>	480	3.78	3.01	7.3	11.1	152
	12 <sup>00</sup>						
3	12 <sup>12</sup>	275	3.36	2.20	5.5	7.4	135
	12 <sup>30</sup>						
4	12 <sup>42</sup>	250	2.98	2.03			
	13 <sup>00</sup>						

The blank samples having been taken, inulin and ascorbic acid were injected intravenously: 0.5 gm ascorbic acid in the first 6 experiments and in the remaining 1 gm — of inulin generally 5 gm. The further course of the experiment is apparent from table IV.

## 2. Results

The material, collected from 17 experiments with in all 84 individual determinations, is combined in table V. The given values for *S* have been corrected to be valid for a body size of 1.73 m<sup>2</sup> according

Tab. V.

Results of comparative investigations on plasma ascorbic acid and excretion rate after intravenous injection of 500 (No. 200—205) or 1,000 (No. 206—216) mgm ascorbic acid. Material consists of 84 single determinations on 17 healthy individuals. First determination 1 hour after injection, thereafter each 30 minute (cf. tab. IV). Excretion rate corrected to be valid for a body size of  $1.73 \text{ m}^2$  (see foot-note, page 31).  $T_m$  = maximal reabsorptive rate,  $F$  = filtration rate, calculated from the excretion curve, in mgm and ml per minute, resp.

$P_T$  is the value of  $P$  when  $S$  equals 0.

Exp. No.	Plasma conc. mgm per cent ( $P$ )	Diuresis ml/min.	Excreted mgm/min.	Body size corr. fact.	Excreted mgm/min. (reduced) ( $S$ )	$T_m \pm \epsilon(T_m)$	$F \pm \epsilon(F)$	$P_T$
200	2.69	10.7	1.41	0.82	1.16	$2.1 \pm 0.91$	$120 \pm 37$	1.8
	2.40	4.3	0.94		0.77			
	2.40	5.8	0.69		0.57			
	2.14	7.9	0.62		0.51			
201	2.80	14.6	1.65	0.91	1.50	$1.9 \pm 0.29$	$121 \pm 12$	1.6
	2.51	14.6	1.27		1.16			
	2.39	14.9	1.08		0.98			
	2.27	8.2	0.96		0.87			
202	2.73	6.7	1.78	0.98	1.74	$1.6 \pm 0.15$	$121 \pm 7$	1.3
	2.31	7.8	1.26		1.23			
	2.02	9.7	0.96		0.94			
	1.93	8.3	0.76		0.74			
203	2.76	15.1	1.68	0.90	1.51	$1.9 \pm 0.22$	$125 \pm 9$	1.5
	2.47	12.8	1.34		1.21			
	2.30	10.2	1.00		0.90			
	2.18	10.3	0.86		0.77			
	2.06	10.0	0.76		0.68			
204	3.28	16.3	2.24	0.96	2.15	$1.7 \pm 0.23$	$115 \pm 8$	1.4
	2.90	16.2	1.73		1.66			
	2.64	15.7	1.41		1.35			
	2.35	14.3	1.13		1.08			
205	2.90	6.8	1.55	0.91	1.41	$2.3 \pm 0.62$	$127 \pm 24$	1.8
	2.52	14.3	1.09		0.99			
	2.44	7.6	0.76		0.69			
	2.27	6.8	0.71		0.65			

(Tab. V, cont.)

206	4.60	14.2	4.35	0.97	4.22	$2.6 \pm 0.25$	$149 \pm 8$	1.8
	3.62	8.8	2.85		2.76			
	3.15	10.5	2.35		2.28			
	2.90	10.3	1.64		1.59			
	2.77	10.8	1.44		1.40			
	2.56	7.1	1.29		1.25			
207	3.57	11.5	3.00	0.96	2.88	$2.1 \pm 0.50$	$139 \pm 17$	1.5
	3.11	14.5	2.45		2.35			
	2.98	15.3	2.08		2.00			
	2.65	14.9	1.78		1.71			
	2.60	10.7	1.54		1.48			
208	4.36	7.8	3.38	0.90	3.04	$2.5 \pm 0.23$	$124 \pm 7$	2.0
	3.82	8.4	2.33		2.10			
	3.52	11.3	2.08		1.87			
	3.28	12.3	1.76		1.58			
	3.06	11.3	1.44		1.30			
	2.90	12.4	1.24		1.12			
	2.77	11.7	1.11		1.00			
209	3.86	10.2	3.59	0.81	2.91	$2.4 \pm 0.35$	$135 \pm 12$	1.8
	3.32	6.0	2.44		1.98			
	2.94	10.0	1.83		1.48			
	2.73	8.4	1.46		1.18			
	2.44	10.3	1.36		1.10			
	2.49	9.2	1.12		0.91			
210	4.33	8.3	4.77	0.93	4.44	$2.6 \pm 0.27$	$163 \pm 8$	1.6
	3.61	8.1	3.41		3.17			
	3.06	11.2	2.60		2.42			
	2.69	11.5	2.08		1.93			
	2.69	10.5	1.81		1.68			
	2.56	9.7	1.51		1.40			
211	3.83	11.7	2.48	0.97	2.41	$2.0 \pm 0.58$	$115 \pm 17$	1.6
	3.48	11.2	1.91		1.85			
	3.15	11.5	1.74		1.69			
	2.98	11.4	1.39		1.35			
212	3.86	19.0	3.46	0.83	2.87	$2.8 \pm 0.48$	$143 \pm 16$	2.0
	3.40	15.2	2.32		1.93			
	3.11	10.3	1.78		1.48			
	2.86	10.3	1.53		1.27			
	2.60	6.8	1.19		0.99			
	2.44	12.7	0.94		0.78			

(Tab. V, cont.)

213	3.57	8.8	2.50	0.90	2.25	$1.9 \pm 0.07$	$116 \pm 2$	1.6
	3.11	8.7	1.92		1.73			
	2.73	5.7	1.45		1.31			
	2.60	6.0	1.22		1.10			
	2.44	3.3	1.07		0.96			
214	4.41	12.3	3.91	0.86	3.36	$1.9 \pm 0.55$	$119 \pm 15$	1.6
	3.78	16.0	3.01		2.59			
	3.36	9.2	2.20		1.89			
	2.98	8.3	2.03		1.75			
215	4.03	11.1	3.73	0.94	3.51	$1.8 \pm 0.40$	$133 \pm 14$	1.4
	3.40	6.2	3.00		2.82			
	2.90	12.2	2.43		2.28			
	2.77	14.9	1.98		1.86			
	2.60	13.1	1.56		1.47			
	2.31	9.4	1.41		1.33			
216	3.96	14.0	3.22	0.98	3.16	$2.6 \pm 0.15$	$144 \pm 5$	1.8
	3.12	13.5	1.89		1.85			
	2.59	11.8	1.28		1.25			
	2.35	6.5	0.81		0.79			

to principles mentioned above. The values of the plasma concentration vary, as will be seen, from between 4.60 and 1.93 mgm per cent and the values of the excretion rate between 4.44 and 0.51 mgm per minute. The diuresis which through an iterated water administration is kept as constant as possible, shows, in spite of this, relatively large variations in some cases but no correlation between the diuresis and any other registered factor has been observed.

**Statistical treatment.** In order to judge whether the excretion curve at the here employed plasma concentrations is straight and hence applicable for determination of  $T_m$  and  $F$ , the author has subjected the combined material to a statistical examination and calculated the equation for the straight line of regression according to the general formula

$$y = a_0 + a_1 x; \quad (4)$$

where  $a_0$  and  $a_1$  are the regression constants. In the present case  $x$  represents the plasma concentration ( $P$ ) and  $y$  the excretion rate ( $S$ ).



One must, however, be positive that the material is suitable for a combination. In calculating the straight lines of regression for each individual experiment the author has obtained the regression constants and their mean errors reproduced in table VI. From these appears that the various lines of regression have, on the whole, a similar course, wherefore it seems justifiable to combine the material

*Tab. VI.*

Regression constants and their mean errors in calculating the straight lines of regression for each individual experiment.

No.	$n$	$a_0 \pm \varepsilon(a_0)$	$a_1 \pm \varepsilon(a_1)$
200	4	$-2.14 \pm 0.908$	$1.20 \pm 0.371$
201	4	$-1.89 \pm 0.292$	$1.21 \pm 0.117$
202	4	$-1.55 \pm 0.147$	$1.21 \pm 0.069$
203	5	$-1.87 \pm 0.219$	$1.25 \pm 0.093$
204	4	$-1.65 \pm 0.230$	$1.15 \pm 0.082$
205	4	$-2.29 \pm 0.615$	$1.27 \pm 0.242$
206	6	$-2.62 \pm 0.252$	$1.49 \pm 0.080$
207	5	$-2.05 \pm 0.504$	$1.39 \pm 0.168$
208	7	$-2.50 \pm 0.230$	$1.24 \pm 0.065$
209	6	$-2.41 \pm 0.346$	$1.35 \pm 0.115$
210	6	$-2.64 \pm 0.265$	$1.63 \pm 0.083$
211	4	$-2.03 \pm 0.576$	$1.15 \pm 0.173$
212	6	$-2.80 \pm 0.476$	$1.43 \pm 0.155$
213	5	$-1.88 \pm 0.065$	$1.16 \pm 0.022$
214	4	$-1.92 \pm 0.545$	$1.19 \pm 0.148$
215	6	$-1.78 \pm 0.400$	$1.33 \pm 0.135$
216	4	$-2.56 \pm 0.150$	$1.44 \pm 0.051$

from the individual experiments to one single large population ( $n = 84$ ) and to calculate the straight line of regression for the entire material. The equation is thus calculated

$$y = -2.04 + 1.26x; \quad (5)$$

where the regression constant  $a_0 = -2.04 \pm 0.19$  and  $a_1 = 1.26 \pm 0.06$ , while the correlation coefficient is  $+0.91 \pm 0.018$ .

If, however, an attempt is made to smooth the material with a parabola of second degree no significant curvature is obtained.

Ascorbic acid  $T_m$ . From the statistical computation it thus appears that the excretion curve for ascorbic acid within the plasma con-

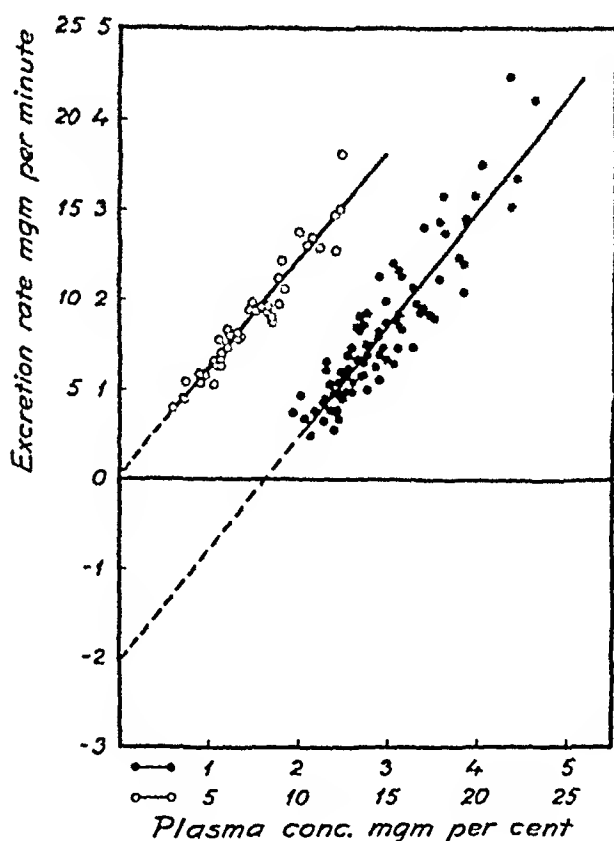


Fig. 17. Excretion curve for inulin (left) and ascorbic acid (right) at falling plasma concentrations in one and the same material.  $n = 40$  and  $84$ , resp. Equations of the regression lines:

$$S_{In} = 0.22 + 1.21 P_{In}, \text{ resp. } S_{HAsc} = -2.04 + 1.26 P_{HAsc}.$$

centration zone in question may be considered straight. In fig. 17, where all the individual determinations are furnished, the position of this line of regression is reproduced. On scrutiny of the position of the individual points around this line one is, however, struck by the fact that the uppermost points are rather far outside and to the left of

the line, which may lead to the suspicion that the line has a slight curvature upwards at high values of plasma concentration. In order to further investigate whether this really is the case the excretion curves for the experiments to which the involved points belong have

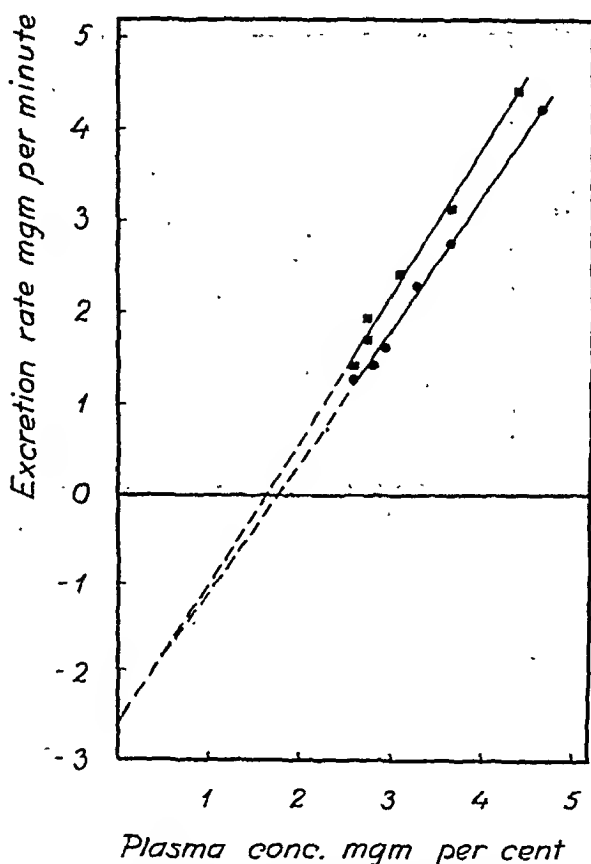


Fig. 18. The excretion curves of exp. 206 and 210. Equations of the regression lines:  $S_{HAsc} = -2.6 + 1.49 P_{HAsc}$ ; and  $S_{HAsc} = -2.6 + 1.63 P_{HAsc}$ .

been reproduced separately (fig. 18). As will be seen there is no possibility of letting the regression lines of these points be a curved one, as the straight line also here smooths the points satisfactorily.

The involved points' position outside the smoothing line is instead rather due to the fact that the two individual smoothing lines in question (experiments 206 and 210) have less inclination — i.e. have a higher  $a_1$  — than the majority of the others.

The straightness of the excretion curve within the plasma concentration zone 4—2 mgm per cent at falling plasma concentrations must, however, be considered as a strong support for the assumption of a constant, of variations in the plasma concentration independent, reabsorptive rate. *With the present methodics the proposed working hypothesis thus apparently holds good and the requirements are found for determining the value of the maximal reabsorptive rate, ascorbic acid  $T_m$ , and filtration rate,  $F$ , according to the previously outlined method.*

According to the calculations, page 18, the regression constant  $a_0$  is the numerical expression for  $T_m$ , whereby  $T_m$  will be expressed in the same measure as the excretion rate, i. e. in mgm per minute. Thus for the entire material ( $n = 84$ ) ascorbic acid  $T_m = 2.0 \pm 0.19$  mgm per minute.

It has been pointed out that the values have been corrected according to above mentioned principles to hold good for an "ideal" body size of 1.73 m<sup>2</sup> and that the correction factor obtained has been multiplied with the excretion rate, which in turn leads to a correction of  $T_m$  and  $F$ . Now  $T_m$ , however, is practically not at all influenced by such a correction, as  $T_m$  for the uncorrected material gives the value  $2.2 \pm 0.18$ . It immediately appears that the difference between this value and the value for the corrected material is inconsiderable and definitely not significant. This is best explained by the fact that the correction factor generally lies very close to 1 with a mean value of 0.91.

The size of the value of the maximal reabsorptive capacity shows an extremely good accordance with the corresponding value determined at concentration balance. As earlier mentioned SHERRY et al. (1940) have investigated the maximal reabsorptive rate at constant supply of ascorbic acid and at concentration balance. It was thereby found that the human kidney was capable of reabsorbing 1.2 to 2.1 mgm of ascorbic acid with an average of 1.7 mgm per 100 ml of glomerular filtrate, or — with a filtration rate of, say, 120 ml per minute — a capacity of about 2 mgm per minute.

In 13 of the experiments or during altogether 40 periods of excretion the filtration rate was determined by the aid of inulin clearance (table VII). Here, however, an opportunity is afforded to determine the reabsorptive rate for each period according to the equation (2), through the value of the plasma concentration, the filtration rate, and the excretion rate. In such a determination, it appears that the

Exp. No.	Body size corr. fact.	Diuresis ml per minute	Inulin		
			Plasma conc. mgm per cent	Excreted mgm per minute	Clearance ml per minute (reduced)
201	0.91	14.6	10.4	14.4	126
		14.6	8.9	12.3	126
		14.9	7.4	10.9	134
202	0.98	6.7	9.0	12.5	136
		7.8	5.6	7.0	123
		9.7	4.8	6.0	123
204	0.96	16.3	12.3	18.8	147
		16.2	9.2	11.2	117
		15.7	6.6	8.5	124
		14.3	5.4	6.7	119
205	0.91	6.8	8.3	10.7	117
		14.3	6.0	8.2	124
		7.6	3.7	6.0	148
206	0.97	14.2	12.3	15.6	123
		8.8	8.5	9.4	107
		10.5	5.6	8.1	140
207	0.96	11.5	11.1	13.4	116
		14.5	7.5	10.0	128
		15.3	5.6	7.3	125
		14.9	4.5	6.2	132
208	0.90	7.8	12.0	14.3	107
		8.4	8.2	10.4	114
		11.3	6.0	9.3	139
209	0.81	10.2	7.6	11.7	125
		6.0	5.6	9.8	105
		10.3	3.0	4.9	132
210	0.93	8.1	6.2	8.7	131
		11.2	4.5	5.8	120
		11.5	3.6	4.9	127
211	0.97	11.7	7.9	10.1	124
		11.2	5.3	6.6	121
212	0.83	19.0	10.0	16.7	139
		15.2	8.6	10.8	105
		10.3	5.3	8.0	125
214	0.86	12.3	12.0	17.2	123
		16.0	7.3	11.1	131
		9.2	5.5	7.5	116
215	0.94	11.1	10.7	14.4	127
		6.2	8.9	10.4	110
		12.2	6.6	8.5	121

## VII.

tion rate and filtration rate (inulin clearance). Regarding the reduction of the values mental technic see tab. IV, page 52.

Ascorbic acid				Notes
Plasma conc. mgm per cent	Excreted mgm per minute (reduced)	Filtered mgm per minute (reduced)	Reabsorbed mgm per minute (reduced)	
2.80	1.50	3.53	2.03	Only 3.5 gm inulin inj.
2.51	1.16	3.16	2.00	
2.39	0.98	3.20	2.22	
2.73	1.74	3.71	1.97	
2.31	1.23	2.84	1.61	
2.02	0.94	2.49	1.55	
3.28	2.15	4.82	2.67	
2.90	1.66	3.39	1.73	
2.64	1.35	3.27	1.92	
2.35	1.08	2.80	1.72	
2.90	1.41	3.39	1.98	Only 3 gm inulin inj.
2.52	0.99	3.13	2.14	
2.44	0.69	3.61	2.92	
4.60	4.22	5.66	1.44	
3.62	2.76	3.87	1.11	
3.15	2.28	4.41	2.13	
3.57	2.88	4.14	1.26	
3.11	2.35	3.98	1.63	
2.98	2.00	3.73	1.73	
2.65	1.71	3.50	1.79	
4.36	3.04	4.67	1.63	Only 4 gm inulin inj.
3.82	2.10	4.36	2.26	
3.52	1.87	4.89	3.02	
3.86	2.91	4.83	1.92	
3.32	1.98	3.49	1.51	
2.44	1.10	3.22	2.12	
3.61	3.17	4.73	1.56	
3.06	2.42	3.67	1.25	
2.69	1.68	3.42	1.74	
3.83	2.41	4.75	2.34	
3.48	1.85	4.21	2.36	
3.86	2.87	5.37	2.50	
3.40	1.93	3.57	1.64	
3.11	1.48	3.89	2.41	
4.41	3.36	5.42	2.66	
3.78	2.59	4.95	2.36	
3.36	1.89	3.90	2.01	
4.03	3.51	5.12	1.61	

reabsorptive rate of the entire material shows variations between 0.9 and 3.0 mgm per minute, but that these variations generally are conditioned by variations in inulin clearance. As we, however, do not know the absolute method error in determining the inulin clearance, no conclusions can be drawn from a possible correlation between the filtration rate and the reabsorptive rate. No positive correlation between the plasma concentration and the reabsorptive rate for ascorbic acid is to be found. The extension of  $P$  in these experiments is, however, too small to attribute it any importance in these cases. The mean value for the reabsorptive rate, determined in this manner will be  $1.9 \pm 0.08$  mgm per minute ( $n = 40$ ) and no positive difference between this value and that previously obtained by determination through ascorbic acid  $T_m$  will be found.

**Filtration rate.** According to the working hypothesis the filtration rate —  $F$  — may be determined directly from the excretion curve for ascorbic acid (p. 18), inasmuch that at a constant filtration rate the amount of filtered ascorbic acid per unit time is directly proportional to the plasma concentration. At a constant reabsorptive rate the filtration rate will be expressed by the inclination of the excretion curve, which in turn may be expressed by  $\text{tg } \alpha$ , (Fig. 1, p. 19) or by the regression constant  $a_1$ . If thereby  $P$  is expressed in mgm per cent and  $F$  in ml per minute then the value of  $F$  in ml per minute will be obtained by the multiplication of  $a_1$  with 100. From equ. (5) is then obtained the mean filtration rate for the entire material equal to  $126 \pm 6$  ml per minute. (For the uncorrected material =  $138 \pm 6$  ml per minute; difference  $12 \pm 8$ , and thus not significant.)

This value, which lies between the mean values for inulin clearance found by HOGEMAN (1943) and by JOSEPHSON and LINDAHL (1943) does not differ significantly from either of these values. Neither does it differ from the here calculated mean value for inulin clearance, as derived from the excretion curve for inulin (fig. 17). The equation for this curve is namely:

$$S_{In} = 0.22 + 1.21 P_{In}; \quad (6)$$

As the regression constants will be  $0.22 \pm 0.44$  resp.  $1.21 \pm 0.06$ , it may be seen for one thing that the line extended towards the  $x$ -axis has no significant deviation from zero, and for another that

the filtration rate, for the entire material averages  $121 \pm 6$  ml per minute, which same value is in close agreement with, and in no wise significantly different from, that calculated from the ascorbic acid curve.

*The value of  $F = 126 \pm 6$  ml per minute — calculated from the ascorbic acid curve may thus apparently well be used as an absolute measure for the filtration rate in glomeruli.*

Ascorbic acid  $P_T$ . The intersection of the extended excretion curve and the x-axis, i. e. the value of  $P$  when  $S$  equals 0 is of interest in that this value —  $P_T$  — indicates the relation between the kidneys' total reabsorptive capacity and the total filtrative rate. It is, in other words, the theoretical threshold value of the kidneys and amounts on an average to 1.6 mgm per cent. From table V it further appears that this value is fairly constant in different individuals, only varying between 1.3 and 2.0 mgm per cent.

### 3. Discussion

The established straightness of the excretion curve at falling plasma concentrations within the present concentration zone, and the values obtained for the reabsorptive rate and the filtration rate reveal that it in principle is possible to apply the methodics as a combined renal function test. The practical availability, on the other hand, will naturally be dependent on the size of the standard error of the method. It is evident that the method described above, the immediate purpose of which was to establish the form of the excretion curve — regards at the same time being taken to the simultaneous inulin clearance determination — is not to be considered as direct suitable as function test.

It is, however, of the greatest importance for the continued elaboration of the methodics to know the error of the present method, thus particularly the error of the individual observations and the point at which the limits for the physiological variation should be drawn, and also, as far as possible, to recognize the factors contributing to the source of this error. It is thereby not so much the relation between the variants  $P$  and  $S$  (table V) which is of interest, but rather the variation of the two regression constants, giving the



values of  $T_m$  and  $F$ . Under such circumstances it seems justifiable to determine the mean values and the mean errors of the individual  $T_m$  and  $F$  in the usual way, on the basis of the values obtained for the regression constants.

It appears from the table that the number of observations on which the individual  $T_m$  and  $F$  are based, vary, and that the mean errors for the individual  $T_m$  and  $F$  also vary. It may accordingly seem justifiable to attach differing weights to the different values of  $T_m$  and  $F$ . As appears from table VIII, however, it is of no importance whether calculating with weighted or not weighted means and we may, by

*Tab. VIII.*

Mean value of  $T_m$  and  $F$  calculated from weighted and not weighted means (tab. VI, page 56). In the first case the same weight is attached to each individual value of  $T_m$  and  $F$ . In the second case the values of  $T_m$  and  $F$  are weighted with the number of observations on which are based each individual value. In the third case consideration has been taken to the mean error of each individual value.

$n$	$T_m$		$F$	
	$M \pm \epsilon(M)$	$\sigma$	$M \pm \epsilon(M)$	$\sigma$
17	$-2.15 \pm 0.095$	$\pm 0.39$	$130 \pm 3.5$	$\pm 14$
84	$-2.19 \pm 0.095$	$\pm 0.39$	$131 \pm 3.7$	$\pm 15$
342	$-2.06 \pm 0.097$	$\pm 0.40$	$131 \pm 4.0$	$\pm 16$

way of example, for the further discussion adopt a mean reabsorptive capacity of 2.1 mgm per minute and a mean filtration rate of 131 ml per minute. The calculated standard deviation for these will be approximately  $\pm 0.4$  and  $\pm 15$ , respectively and the border values for the maximum reabsorptive capacity ( $\pm 3\sigma$ ) will lie at approximately 3.3 and 0.9 mgm per minute and for the filtration rate at 176 and 86 ml per minute.

The error found may seem great, particularly as regards the error of  $T_m$ . This, however, is to some extent explained by the position of  $T_m$  in relation to the median point of the curve (see graph, fig. 18, p. 58).

Before attempting to analyze the cause of the systematic errors contributing to the standard error, it should be stressed that the straightness obtained evidently does not exclude the possibility of

real variations in the reabsorptive rate or in the filtration rate, variations which thus are concealed in the found standard error. We have, however, no possibility whatsoever, if actually there be variations, of directly ascertaining their values and must therefore instead to the furthest possible extent clear up other disturbing factors contributing to the source of standard error, subsequently establishing whether or not it is significantly influenced thereby.

It should first of all be possible to diminish the systematic error in analysis, thereby including the error arising in the analysis of blood samples. That such an error occurs is *inter alia* evident from i. e. the experiments 200 and 209 (p. 53). It is probable that these to the greatest part are rather due to the haste in taking samples, occasioned by the inulin clearance determination simultaneously done, than to errors committed in the chemical determination of the ascorbic acid.

Those simplifications of the methodologies which always must be made in computations of the excretion rate at falling plasma concentrations are with certainty the greatest source of error. The demand that the test subjects at the exact time and completely are to empty the bladder will often be difficult to fulfill; there may be a time displacement of one or several minutes and urine may remain in the bladder from one period to the next. The error arisen in one period, however, is generally compensated for in the next period, which in certain instances may explain the fair mean value of the regression constants in spite of the large mean errors. This error can hardly be eliminated, but it diminishes with the number of determination periods. It also appears from table V that, as was expected, the mean error generally is considerably larger in experiments with but 4 periods of excretion than with 5 to 7. It therefore at present seems indispensable to maintain a requirement of approximately 6 periods of determination.

The division of the investigation into half-hour periods brings about that the points on the excretion curve do not lie equally distributed along it, lying closer the more the plasma concentration falls; this in turn brings about that the various points have unequal weight in determining the line of regression. As is seen from table V the mean difference between the plasma concentration in the first and second periods will approximately be 0.5 mgm per cent, while it for instance in the fourth and fifth period only will be approximately one-half

that amount. In order to attain a more uniform distribution of the points, thereby diminishing the systematic error, the length of the periods should successively be increased.

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The methodics for determination of the tubular reabsorptive capacity and the glomerular filtration rate in man, the premises of which we have studied in the present investigations, and the application of which on physiological and clinical material will be discussed in another connection, have, in accordance with the argumentation stated above, the following appearance:

The day prior to the investigation a minimum of 2 gm of ascorbic acid is administered perorally, and one hour prior to the beginning of the experiment, 1 gram is further given intravenously. Peroral water administration, in order that the diuresis if possible may lie at approximately 5 ml per minute. One hour following injection the bladder is emptied, voluntarily or by catheter, after which the urine is collected during 6 periods of time of increasing length, i. e. approximately 20, 20, 25, 30, 35, and 40 minutes. Approximately 3 minutes before the middle of each period a sample for the plasma ascorbic acid determination is taken. Analyses and calculation of  $T_m$  and  $F$  according to the methodics described above.

#### 4. Summary

In the present section it has experimentally been established:

1. That the excretion curve for ascorbic acid at falling plasma concentrations within the concentration range 4—2 mgm per cent may be considered straight, the conditions required for calculation the maximum reabsorptive capacity and the filtration rate thus being present.
2. That the thus calculated reabsorptive capacity of 17 healthy test subjects had a mean value of  $2.1 \pm 0.10$  mgm per minute.
3. That the simultaneously calculated filtration rate was  $131 \pm 4$  ml per minute, these values individually being in good agreement with previous and by other methodics obtained values.

The above (Section II) propounded working-hypothesis thus seems to be experimentally proven, the premises for simultaneously determining the glomerulus function and the proximal tubule function from the ascorbic acid excretion curve accordingly being found.

## VI

### On the theories of the reabsorption mechanism

Although many investigations still will be required before we have a definite picture of the quantitative conditions at renal excretion of ascorbic acid, we already have a possibility of forming a conception as to how this excretion on the whole takes place. On the basis of earlier and present data it should be feasible to reconstruct the excretion curve under different conditions in approximately the following manner:

1. At *concentration balance*, i. e. in determining the excretion rate after the plasma concentration has been constant for some time, the excretion rate is very low but rising at plasma concentrations between 0 and approximately 1.5 mgm per cent. Above this value the curve rises suddenly, becoming straight at an individually variable higher value.

The form of this curve is satisfactorily explained under the assumption of a constant total filtration rate and an absolutely limited, total reabsorptive capacity, assuming that the relation *filtration rate/reabsorptive capacity* is variable for different nephrons.

2. At *continuously rising plasma concentrations* the course of the curve is on the whole analogous to that at concentration balance, with the exception that the marked increase in the excretion rate sets in at a higher plasma concentration value.

This should be due to the storage of ascorbic acid in the kidney cells, this ascorbic acid not being given off to the circulation.

3. At *falling plasma concentrations* the excretion curve above approximately 2 mgm per cent is straight, but its further course is as yet questionable. Certain observations, however, indicate that it is in agreement with the curve at concentration balance.

According to this we should thus in the tubules have two distinct reabsorptive mechanisms, the one supplying ascorbic acid to the tu-

bules own cells, the other distributing the large amount of filtered ascorbic acid to the entire organism.

Regarding the laws governing the former process we are as yet ignorant, but we have reason to assume, as pointed out in the foregoing, that it takes place in approximately the same manner as does the reabsorption of ascorbic acid in the other cells of the body. It is also reasonable to assume that this reabsorption is limited in as much that only a certain amount of the substance is absorbed. At saturation this reabsorption should accordingly not be a source of error in quantitative determinations of the other reabsorptive process.

As to the specific reabsorptive process, on the other hand, directed towards giving off the filtered ascorbic acid to the circulation, we can with some certainty determine *that* it is associated to a definite part of the tubules, i. e. the proximal part, *that* it has an absolute maximal limitation, and *that* finally, before the maximum capacity is fully reached, the reabsorption is practically complete.

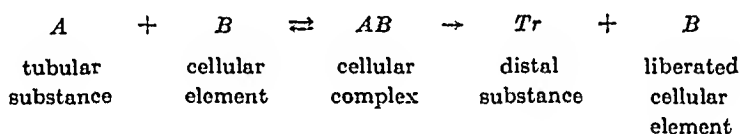
It seems at present out of question to draw any definite conclusions in regards to the reabsorptive mechanism from the present data. It has, however, repeatedly been stressed that ascorbic acid, as regards its tubular reabsorption, behaves much as glucose, and we may therefore consider it justifiable to transfer our experience from the latter substance to apply also to the former. It has further been pointed out that the tubular excretion also quantitatively seems to mirror the reabsorption and we may thus possibly gather information also from this process.

In regards to glucose the fact that urine normally is practically free from glucose indicates that the substance in question must be reabsorbed against a concentration gradient. This is, however, by no means a surprise, as it has been possible to demonstrate that the intestinal reabsorption of glucose also takes place against a concentration gradient (VERZÁR, 1935; BÁRÁNY and SPERBER, 1939). The reabsorption must therefore be regarded as an active process and the energy for this obtained from a local cellular metabolism.

It is true that glucose appears to be that carbohydrate most avidly reabsorbed, but the reabsorptive mechanism does not seem to select particularly this specific and for the body indispensable carbohydrate, other hexoses and even pentoses also apparently being reabsorbed. Thus, HÖBER (1933) in perfusion experiments in frog found the following order of reabsorptive rate: glucose>galactose>mannose

>fructose>xylose>arabinose. Now not only does a difference in the reabsorptive rate seem to be present at exclusive administration of the various substances in question, but it has also been demonstrated that i. e. glucose completely and reversibly blocks the reabsorption of xylose (SHANNON, 1938), which gives us reason to suspect that the mechanism is common for these substances. Analogous observations have also been made regarding glycine and creatine (PITTS, 1943) and others.

In endeavouring to explain this phenomenon and also the fact that the reabsorption has a fixed upper limit SHANNON (1939 and 1942) — originally for the opposite condition in tubular excretion — built up a theory of the tubular mechanism, basing it on a hypothetical chemical process in the tubule cells. He assumes, first, that in the sequence of reactions that result in its transfer, the solute enters into reversible combination with some cellular element which is present in a limited amount, and, second, that the decomposition of this complex limits the further progress of the solute. Thus there are required two consecutive reactions:



In order to arrive at a maximal rate of transfer under these circumstances, the second process must be a first order process, its rate slow in relation to the rate of attainment of equilibrium in the first.

In order to explain the fact that certain substances block the reabsorption of other substances he further assumes that the component *B* is common for these but that the one substance excludes the other in the competition for this carrier substance. As regards phlorizin's capacity completely to block the reabsorption of glucose he suggests that this is due to the fact that phlorizin enters into competition with glucose for the transport mechanism and displaces glucose from it in much the same way that glucose can exclude xylose.

Shannon's hypothesis may undoubtedly be a satisfactory explanation of the absolute limitation of the reabsorptive rate and possibly also of the fact that one substance blocks the reabsorption of another.

but it says nothing of how the substance dissolved in tubular fluid comes into definite contact with this carrier substance.

Respecting this question we can as yet only make conjectures. The division of the renal process on a large number of nephrons, and the tubules' internal surface, large in relation to the lumen, evidently decrease the distance between the molecules of the solute and the cell surface. As, moreover, electrical potential differences across the tubular wall are considered demonstrable (KELLER, 1933; WILBRANDT, 1938) attempts have been made in drawing up a relatively detailed theory of the tubular transfer work with these potential differences as sources of energy (EKEHORN, 1938).

In endeavouring further to explain how the substance dissolved in preurine comes into contact with the tubule wall, thus becoming amenable to reabsorption, however, it seems to be of some interest to undertake a comparison between the tubule work and an adsorption analysis *in vitro*.

The external conditions for such a comparison are unquestionably found in the form of the tubules' internal surface, enormous in relation to the tubule lumen, and very well adapted as adsorbent. To this surface would take place a relatively rapid adsorption, the quantitative conditions of which would be determined by the adsorption isotherm and the qualitative conditions by the specific properties of the adsorbent.

As regards the former it is noteworthy that adsorption isotherms with an absolute maximum frequently are found at *in vitro* experiments, and it is naturally tempting to endeavour to explain the absolute limitation of the reabsorptive rate on the basis of such an adsorption isotherm. To switch over from the quantitative limitation in the latter case to the limitation of rate in the former case, however, seems for the present impossible. A comparison between the qualitative conditions *in vivo* and *in vitro* seems to be more profitable.

*In vitro* the capacity of adsorption is dependent on the adsorbent and the solution, but also to a great extent on the molecular structure of the adsorbate, inasmuch that e. g. in certain cases substances with more polar groups, higher molecular weight, etc., are more readily adsorbed. If the solution contains various different adsorbates with unequal affinity to the adsorbent, those with a greater affinity will supersede the others from adsorption.

If analogous laws were valid regarding the tubules certain substan-



ces dissolved in the filtrate would thus accordingly impede others from concentration on the cell surface-adsorbent, thus preventing them from establishing contact with the carrier substance in the cell. Applied to the tubular apparatus this would, for instance, explain the blocking of the xylose reabsorption brought about by an augmentation of the glucose concentration and possibly also the blocking of the glucose reabsorption by phlorizin. By interpreting this blocking as a displacement adsorption the intracellular process assumed by Shannon may be simplified and made less specific, in that the competition for the carrier substance is determined already on the cell surface.

A support for the assumption that the physical forces on the cell surface are of great importance for the choice of substances for tubular transfer is offered in recently published works by HÖBER (1945). In perfusion experiments on frog he has been able to study the active transfer of certain sulfonic acid dye stuffs through the tubule wall, thus establishing that the ability to participate in this process largely depends upon a polar-nonpolar molecular configuration. This should involve a special orientation of the molecule on the cell surface, the orientation in turn being pre-requisite for the further transfer through the cell.

The investigations in this field are evidently as yet too limited to be able to draw any general conclusions as to the importance of the molecular storage on the cell surface for the tubular transfer. However, it seems to be possible through further investigations on the same lines to explain various processes taking place in tubular transfer according to known physical laws, thus lessening the specificity which has been attributed to the tubule cells.

*Summarily*, we may consider, in regards to the tubular reabsorptive mechanism, *that* the reabsorption occurs through an active cellular process of some unknown nature; *that* this process for ascorbic acid is connected with the proximal part of the tubules, and *that* the limitation of the reabsorptive rate may satisfactorily be explained by the occurrence within the tubule cells of a limited amount of carrier substance. It may further be assumed that our *general laws of adsorption to a certain extent are applicable to the system of the tubular cell surface — tubular fluid*, and that certain of the processes previously considered to take place in the tubule cells may be assumed to take place already on the cell *surface*, according to known physical laws.

## VII

### Summary

The renal excretion curve for ascorbic acid, i. e. the excretion rate at different plasma concentrations, has been studied under certain physiological conditions in man. The investigation has given the following results:

1. The excretion rate at rising plasma concentrations between 0 and approximately 1.7 mgm per cent is very low, amounting at maximum to 0.07 mgm per minute.

2. Between approximately 1.7 and 2.5 mgm per cent the excretion curve rises suddenly — the threshold zone — subsequently becoming straight. The values, however, show wide individual variations.

3. The threshold zone lies higher at rising than at falling plasma concentrations.

4. The excretion curve is to some extent reproducible in one and the same individual.

5. The established characteristics of the excretion curve may be explained by the assumption of a constant filtration rate and an absolute limited reabsorptive capacity, also assuming that the relation *filtration rate / reabsorptive capacity* is different for different nephrons and that the kidney itself at rising plasma concentrations stores ascorbic acid. (Section IV)

6. The excretion curve at falling plasma concentrations between 4 and 2 mgm per cent may be considered straight. From this curve may be calculated the filtration rate in the glomeruli as well as the tubules' maximal reabsorptive capacity.

7. The thus calculated mean filtration rate and mean reabsorptive capacity was for 17 healthy subjects  $131 \pm 4$  ml per minute and  $2.1 \pm 0.1$  mgm per minute, respectively. As these values are in good agreement with previous and by other methodics obtained values for filtration rate and reabsorptive capacity, the premises for a

combined renal function test for determining the glomerulus function and the proximal tubule function are thus on hand. (Section V)

8. The reabsorption of ascorbic acid as well as of glucose and other substances reabsorbable in the proximal tubules supposedly takes place as follows: After filtration in the glomeruli these substances are concentrated through a process of adsorption on the tubular cell surface and seized by a special carrier substance — present in a limited amount — in the tubule cells. They are then by an active cellular process transferred through the tubule wall. (Section VI)

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INVESTIGATIONS ON  
THE FAT ABSORPTION IN  
THE INTESTINE





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# INVESTIGATIONS ON THE FAT ABSORPTION IN THE INTESTINE

THE PRESENCE OF FATTY ACIDS AS SOAPS  
IN THE INTESTINAL CONTENT AND THEIR ABSORPTION  
AS PHOSPHOLIPIDS

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*Bengt Strömberg,*  
Prodekan.

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## PREFACE

The work dealt with in the present volume was carried out partly at the chemical department of the Carlsberg laboratory, partly at the zoophysiological laboratory of the University of Copenhagen. The leaders of these laboratories, professors dr. *K. Linderstrom-Lang* and dr. *P. Brandt Rehberg*, have always accorded to me the most cordial hospitality and help in every respect.

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Mr. *A. Nærland*, C. E., Trondheim, has given me permission to publish some of his results concerning the dissociation of fatty acids.

The present work appears as part of a thesis for the doctor's degree at the University of Copenhagen. Two previous papers have been accepted for valuation by the faculty of science (Schmidt-Nielsen 1942 and 1944).

## INTRODUCTION

### IS HYDROLYSIS NECESSARY FOR FAT ABSORPTION OR NOT?

Since the beginning of this century the conception of the digestion and absorption of fats has been dominated by the theories proposed by *Pflüger* (1900, 1).

Investigations up to that time had not settled the questions of the mechanism of fat absorption. The common view was that fats might be taken up by the intestine as fine droplets or in the split state as fatty acids and glycerol. The investigations of the rôle played by the pancreas juice (lipase) and the bile in the digestion had shown that fat was usually split during the digestive processes, but the possibility of absorption of fat droplets was maintained.

In 1900 *Pflüger* proposed his theory of fat absorption. In a publication dealing with the poisonousness of horse meat, he wrote a chapter summarizing his opinions regarding fat absorption.

This work was in no respect supported by experimental investigations, but *Pflüger* had a very decided point of view and denied the possibility of absorption of fat in the unsplit condition. According to *Pflüger* the absorption of fat was only possible after total hydrolysis, and he claimed that the fatty acids were absorbed in the form of their alkali salts.

*Pflüger* knew that neutral fats are not dissolved by the bile acids, and found this to be one of the facts that showed that fats are only absorbed after hydrolysis. But none of the works quoted by *Pflüger* gives the proof of his contention: "All the fat in the intestine must be hydrolysed before absorption."

One of *Pflüger's* arguments was that *Frank* in 1898 had found that ethyl esters of fatty acids were totally hydrolysed before absorption. They were easily absorbed and it was not possible to find



any traces of the unsplit esters in the chylus. Further *Frank* found, after giving palmitic esters, considerable quantities of tripalmitin in the chylus. *Frank* himself stated that he had "with certainty shown that a synthesis of triglycerides takes place during absorption of fat." It is really a fine support for *Pflügers* theory, but it is not permissible from *Franks* results to conclude that a synthesis of triglycerides is an obligate process in fat absorption, and it is even less legitimate to use *Franks* results as a proof for the total hydrolysis of all fats before absorption.

In two articles *Munk* (1900, 1, 2), who took *Pflügers* work as a personal attack, opposed the theories. *Munk* points out many facts that speak against *Pflügers* opinion, but the experimental work carried out until that time was not sufficient to settle the question.

*Pflügers* answer (1900, 3) in the discussion was not less powerful, he sums up almost all the work made upon the digestion of fat until that time, and his conclusion is of course that his theories are infallible.

The discussion was continued for a period of years during which the personal relations of the authors were subject to not much less interest and criticism than the fats.

An attempt to decide the question was made by *Hofbauer* (1900). He fed animals with fat stained with alcanna or sudan, and found that the fat was taken up into the epithelium of the intestine and the chylus together with the dye. He concluded that the fats were absorbed as stained droplets "since the dyes are insoluble in water and would precipitate if the fats were saponified."

The conclusion from the results was immediately rejected by *Pflüger* (1900, 2) as being incorrect. He showed that the dyes are soluble in glycerol, in the bile, and in soap solutions. The dyes are consequently not precipitated when the fats are hydrolysed in the intestine, they may be taken up together with or independently of the fatty acids. The stained fat may therefore be found in the epithelial cells even if the fat is not taken up as droplets.

In spite of the apparent impossibility to demonstrate the mechanism of absorption by means of stained fats, *Wottom & Zwemer* have, only a few years ago (1939), worked with sudan stained fats. Apparently they have not known the rejection of the method made by *Pflüger*, *Henriques* and others. Their work, therefore, contains no data which can bring a decision of the question, and their con-

clusion that fat droplets pass the cuticle border of the intestinal columnar cells is open to the old objections.

The question was apparently settled by the very convincing experiments made by *Henriques & Hansen* (1900), and since that the main point of *Pflügers* theory has been dominating the textbooks as well as the later experimental work.

*Henriques* mixed equal parts of lard and paraffine, and made an emulsion of the mixture. He demonstrated that the droplets of the emulsion contained equal parts of the constituents. In animal experiments he found that the lard was almost completely absorbed, while the paraffine was quantitatively recovered from the feces. He concluded that the droplets of the emulsion were not taken up by the intestine, and that the fat could only be absorbed after saponification.

The only objection that can be made a priori against *Henriques* experiments is that the melting point of the paraffines he used was not below body temperature.

*Bloor* (1913) carried out further experiments, and found that petroleum hydrocarbons (and wool fat) were not absorbed. The melting point of the petrols was below body temperature, and they were fed emulsified as well as unemulsified. *Bloor* states that "change to water-soluble state seems necessary for absorption."

*Mellanby* (1927, 2) found no absorption of paraffine emulsions from cats intestine. *Channon* and *Collinson* (1929) found some absorption of liquid paraffine when fed for a longer period, as concluded from the observed decrease in the iodine number of the unsaponifiable matter of the liver.

It is easily understood that the experiments carried out by *Henriques* and later confirmed by others, in which unsaponifiable substances of lipid character were rejected by the fat absorption mechanism, dominated the view for many years. Until the very recent work of *Frazer* (1942—1944) the main principles of *Pflügers* hypothesis were generally accepted.

#### ABSORPTION OF SOAPS

One point of *Pflügers* hypothesis has been subject to a change during time. *Pflüger* suggested that the fatty acids produced by the action of the lipase were neutralized by the sodium carbonate of the pancreatic juice, and that they were absorbed in the state of sodium salts. The significance of the emulsification and the hydrolysis of

the fats should only be to prepare them for saponification. *Levites* (1907) found excellent absorption of soaps in fistula dogs. The absorption was always in excess of that of the corresponding fatty acids (stearic, palmitic, or oleic acid and their Na salts were tried).

*Pflüger* suggested that the reaction of the small intestine was alkaline. Later on the actual reaction of the intestine has been determined by more reliable (electrometric) methods, and it has been found that the reaction is actually not alkaline.

It is well known that the soaps are decomposed by acids, and it is commonly stated as a fact that soaps cannot exist below pH 8 or 9. The behaviour of soaps at different pH's of the physiological range was investigated by *Jarisch* (1922), who concluded that in blood and tissues practically no soap is present, and that introduced soap is immediately split up. The fatty acids are liberated in a highly dispersed form, and the colloid solutions of fatty acids might to some extent be stabilized by proteins.

The further consequences of the supposition that soaps cannot exist at the pH of the intestine are to be discussed in a later chapter.

#### THE RÔLE OF BILE SALTS IN FAT ABSORPTION

From numerous investigations we know that the bile is a very important factor in fat absorption. If the flow of bile is suspended by pathological changes or by a ligature, the absorption of fats decreases to a minimum. Addition of bile salts to the food restores the possibility of fat absorption.

The importance of the bile acids for the fat absorption may be regarded as a well established fact. On this basis *Verzar* has advanced his theory of the rôle of the bile acids as taking part in the transport of the fatty acids across the free border of the intestinal epithelial cells.

It is a well known fact that fatty acids are easily soluble in bile as well as in solutions of pure bile salts. Further *Verzar & Kuthy* (1929, 1, 2) found that in the presence of bile salts the fatty acids can diffuse through collodion membranes which are otherwise impermeable to them. They supposed that the fatty acids in the intestine pass through the cell membranes associated with 3—8 molecules of bile acid per molecule of fatty acid. In the epithelial cell the complex is split up, the fatty acids are synthesized to neutral fat which is found as visible droplets, and the bile acids are trans-

ported away by the blood circulation and brought to the liver where they are resecreted in the bile.

If *Verzars* theory is accepted, a rough calculation gives the approximate quantity of bile necessary for absorption of a normal fat meal.

For example, 100 gram fat contains 90 gram (0.35 Mol) fatty acid. The average molecular weight of bile acids may be 500, and supposing four moles bile acid per mole fatty acid, we find 700 gram bile acids necessary for absorption of the fat. The bile acid content of the bile is generally less than 10 per cent, which means more than 7 liter bile. The diurnal bile secretion is generally estimated to be about 0.5—1 liter (e. g. *Brand* 1902).

The theory of a circulation of the bile acids in order to form a vehicle for the transport of fatty acids is commonly accepted in text-books, although *Verzar* clearly has taken a standpoint against it (1936, p. 165). He found that the quantity of bile was not sufficient for covering the absorption of fat according to the hypothesis in the form described. Therefore *Verzar* supposed that the bile acids are adsorbed in the epithelial cells, where they form the vehicle necessary for the solubilization and transport of the fatty acids. He says: "When the bile acid — fatty acid complex has entered the epithelial cell, it is immediately broken down. The fatty acid is then synthesized to neutral fat, while the bile acid is free again, and is now adsorbed to the surface of the epithelial layer, where it dissolves more and more fatty acid molecules."

#### ARE THE FATS TRANSPORTED BY THE LYMPH OR BLOOD?

During fat absorption the lymphatics leading from the intestine are white and filled up with a milky lymph containing much emulsified fat. If the thoracic duct is obstructed considerable amounts of fat may still be absorbed (*Frank* 1892). *Frank* (1894) found a fat absorption of 77 per cent when the thoracic duct was tied off, against 96 per cent in normal animals (dogs). The experiments have been repeated by many authors.

The old values for the fat transport by lymph given by *Munk* (1880) (60 per cent by lymph) have been cited again and again and have been regarded as generally valid, but they are based on experimental work which, even if it is in no way unsatisfactory, cannot support a final solution of the question.

*Sulze* (1933) found almost complete absorption of fats in cats

in which the lacteals were tied off. A recent publication of *Little & Robinson* (1941) deals with analyses of the collected thoracic lymph in dogs. They injected cream in the duodenum, and found only 4—17 per cent of the absorbed fat recoverable in the lymph.

Probably the fat which is not found in the lymph passes into the blood stream. Because of the relatively rapid blood flow it is much more difficult to demonstrate by analyses the transport of fat by the blood. In fact the relative increase in the lipid content of blood caused by a certain amount of fat is generally too small for an exact calculation of the quantity of fat.

That an increase does occur in the blood is shown e. g. by *Eckstein* (1925) who found an appreciable though small augmentation of the fatty acid content of blood following the absorption of neutral fat from the duodenum. He used dogs with the thoracic lymph diverted, and controlled his experiments by showing that anesthesia alone caused no increase.

#### RECENT WORK ON FAT ABSORPTION. FRAZERS WORK

The general facts and theories just outlined appear to give a relatively clear view of the main paths of fat absorption, namely:

The relatively unchanged fat passes from the ventricle to the intestine, where it is split up by the lipase of the pancreatic juice, the lipid matter is emulsified and partly kept in solution by the bile components, and the absorption takes place in the form of water soluble complexes of fatty acids. In the epithelial cells neutral fat is resynthesized and transported away partly by the lymph, partly by the blood stream. There is very little evidence to suppose absorption of unsplit fat as droplets.

This common view is not in agreement with the recent work made by *Frazer* during the years 1942—1944.

*Frazers* absorption theory is briefly the following: Fat may be taken up in the intestine in the unsplit condition as a finely dispersed emulsion or in the hydrolysed condition as fatty acids. The unsplit fat passes into the lacteals of the villi and is found as droplets (chylomicrons) in the lymph, the fatty acids pass into the blood vessels and do not take part in forming the fat droplets visible in the villi during fat absorption.

The theory seems to be well founded in the experimental work carried out by *Frazer* and his collaborators.

The very striking theory of absorption of droplets was founded

on an experiment in which *Frazer* (1942) demonstrated an absorption of 60 per cent of paraffine oil when it was finely dispersed. This was as much as the absorption of olive oil in the same period.

In human subjects (1938) *Frazer* found the well known and marked increase in the number of chylomicrons when giving olive oil in a test meal. If the equivalent amount of oleic acid and glycerol was given, there was no rise in the particle curve. If extra lipase was added to the oil meal, there was nearly no rise in the chylomicron curve. It seems to be certain that the particles counted as chylomicrons are really of lipid nature.

Results giving no evidence directly against *Frazer*, but nevertheless of some interest in relation to the chylomicron counts are given by *Little & Robinson* (1941): "The increase in portal plasma lipids during non-lipid as well as lipid absorption suggests a mobilization of stored lipids by the portal blood under the stimulus of absorption itself."

*Frazer's* next publication on the fat absorption (1942) gives the result just mentioned that paraffine oil may be taken up in emulsions. In addition to the chemical evidence of absorption he found droplets of oil in the intestinal wall when the paraffine oil was given as emulsion, but no drops when it was given unemulsified. Further, emulsified paraffine oil caused an increase in the chylomicron count to 200 particles against 20—30 when unemulsified paraffine oil was given.

The particles found in the lymph, in the intestinal wall as well as those of the emulsion are of the order of magnitude of 0.5 micron. *Frazer* suggests that the droplets are made hydrophilic by the 0.2 per cent of oleic acid added before the emulsification, and that they therefore stick to the intestinal wall and can be absorbed.

The partition hypothesis of fat absorption was put forward more clearly, and more results to support it was given in 1943 (*Frazer* 1943, 1—2). *Frazer* could not correlate his findings with the theory of complete lipolysis. The evidence he gives is briefly the following facts:

Absorption of neutral fat is accompanied by milky lacteals, not so fatty acid absorption. Neutral fat gives systemic lipemia and little change in the lipid content of the portal blood while fatty acids cause a marked portal lipemia and relatively little change in the systemic blood. When sudan stained fat was given in moderate doses (2 ml a week) it could be traced to the fat depots and gave no de-

position in the liver. On the other hand fatty acids did not appear in the depots but were deposited in the liver (1943, 1).

Further evidence for the partition theory and the theory that complete lipolysis is not essential in fat absorption was given in the following (1943, 2): Rats fed neutral fat + lipase showed a picture as if fatty acids were ingested. The systemic lipemia following fat absorption in man could be prevented by addition of lipase. If the lipolysis was inhibited by sodium cetyl sulfate, the absorption of triglycerides was not prevented as compared with control animals in the same time.

In the most recent work now available *Frazer* and his coworkers (1943, 3 & 1944, 1—3) investigated the conditions for the spontaneous emulsification of fats. They found that the emulsions from the intestines do not flocculate when lecithinase is added, and conclude that phospholipins are not essential in forming the emulsions. On the other hand the emulsion from blood seems to be stabilized by lecithin since it is flocculated by lecithinase.

The triple system monoglyceride — fatty acid — bile salt was found to form highly dispersed and stable emulsions. They are stable for more than 3 hours at pH 6.0—8.5, the particle size is less than 0.5 micron, and the emulsification takes place almost spontaneously. — Too much stress must not be laid upon these model experiments, but they tell us a great deal about which components are of no and which are of great importance to the emulsification of fats in the intestine. According to *Frazer* one of the chief characteristics of the pancreas lipase should be the power to form monoglycerides for the emulsification systems, the total lipolysis should not be necessary at all for the fat uptake.

In connection with *Frazers* theories some older studies on the problem ought to be remembered, because they give some direct evidence against him.

*Munk* (1880 and 1884) fed his experimental animals on fatty acids prepared from meat, and found the major part of them as neutral fat in the chylus. In dogs killed 3 hours after the administration of fatty acids the lacteals of the mesenterium were filled with milky lymph. Similar results were obtained by *Frank* (1894).

*Franks* results (1898) that ethyl esters of fatty acids are completely hydrolysed in the intestine, and that no traces of them could be found in the chylus, cannot be correlated to *Frazers* theory that fats are only partly hydrolysed without an investigation of the velo-

city at which the different esters are attacked by the lipase. — Another possibility is that, if a part of the ethyl esters were taken up as such, they could be hydrolysed after absorption by the esterases of the serum.

In *Henriques' & Hansen's* investigations (1900) the paraffine — fat emulsion was prepared similar to *Frazers* emulsions. When the hydrocarbons were rejected in these experiments, it might perhaps be due to their high melting point. In the experiments by later investigators (*Mellanby* and others) minute quantities of the hydrocarbons disappeared from the intestinal contents but as very nearly the total amount was refound as non absorbed there was good evidence for ascribing this to unavoidable analytical and technical errors, and to conclude that the hydrocarbons were not taken up.

Experiments on the uptake of hydrocarbons ought to be carried out not only by means of analysis of the intestinal content (as *Frazer* did), but the hydrocarbon absorbed ought also to be traced in the body. If larger amounts of hydrocarbons are taken up, it would be of some interest to see in which way they are treated by the liver where probably they are deposited. *Stetten* (1943) has presented evidence that a hydrocarbon, n-hexadecane, containing heavy hydrogen is efficiently absorbed by the rat. Some of the compound was deposited as such in the tissue lipids and some was converted to fatty acids. If these results are confirmed the uptake of hydrocarbons seems to be indisputably demonstrated. Further, when in absorbing experiments it is not possible to trace the amount of hydrocarbons taken up, they may have taken part in the metabolic processes and have totally disappeared. There is the possibility that the conversion of hydrocarbons to fatty acids may have taken place in the intestine and be caused by bacterial activity. The length of the paraffine chain used by *Stetten* is of the same order of magnitude as those of paraffine oil.

It must be emphasized that *Frazers* work in no way excludes the possibility of a chemical absorption of fats. On the contrary, his experiments with fatty acids (or fats to which ample lipase was added) give results widely different from that of "fat absorption as droplets," and are in good agreement with the conception that chemical processes are essential in the absorption process.

The experimental work made by the writer of this paper, which was planned and carried out before *Frazers* publications reached Denmark, deals with the fatty acids and their absorption. *Frazer* has



thrown much light upon the absorption of unchanged fats, but the writer's results are in no way in opposition to his work.

*Frazers* work makes it possible to explain some of the conflicting or contradictory results of earlier experimental work. As an example *Mellanbys* work (1927, 1) can be mentioned. He found very small quantities of lipase in the pancreatic juice of the cat and no additional lipase in the alimentary tract. When a cannula was inserted into the pancreatic duct, injection of fat emulsion and bile gave rapid absorption of fats into the duodenal lacteals. These results are not explained by the theory of total lipolysis but more easily understood if *Frazers* theory is accepted.

Apparently contradictory results may further be explained by the use of different experimental animals (e. g. the cat has very small amounts of lipase, while in the dog there are ample amounts).

It also seems likely that different parts of the intestine have different capacities for fat uptake. The fat absorption in the large intestine is very small or absent, and also in the ventricle no fat uptake has been demonstrated. *Croner* (1910) experimenting on dogs with Thiry-Vella fistulae found that in the upper parts of the small intestine fats but not soaps were taken up, while the soaps were readily taken up in the lower parts. *Mellanby* (1927, 1) in his experiments just mentioned found rapid absorption of fat emulsions in the duodenum and slow absorption in the ileum of the cat.

There are still facts concerning fat uptake which are not explained either by *Frazers* theory or any other work on fat absorption. *Deuel & Hallman* (1940) investigated the rate of absorption of synthetic triglycerides in the rat. They found that neutral fats containing odd-chain fatty acids are absorbed less than half as rapidly as the corresponding fats containing evenchain fatty acids. The work ought to be repeated with the free fatty acids in order to decide if the phenomenon could be due to the lipase digestion in the intestine, or if it is correlated with the absorption mechanism itself.

#### THE MECHANISM OF FAT ABSORPTION

The uptake of fat as demonstrated by the amounts that disappear from the intestine, or by analysis of the blood, organs etc. give little information about the processes that take place in the intestinal wall during absorption.

Regarding these processes, involved in the transport of fat across the intestinal wall, very little is known. The transport mechanisms

are of an order of magnitude difficult to follow by exact and quantitative methods.

The microscopic picture of fat absorption is well known, as it has been the object of numerous investigations. Some time after feeding fats there is always found droplets of lipid nature at some distance inside the free border of the epithelial cells of the intestine. They are stainable with Sudan or osmic acid, and are commonly regarded as being neutral fat, synthesized in the cells from the fatty acids taken up. The droplets should mostly pass into the central lacteals of the villi, but that the transport of fat may take other routes is already discussed. An extensive review of the histology of fat absorption is given by *Verzar* (1936).

The chemical processes taking place in the cells are not easy to follow. The most promising method is the use of labelled molecules, whose fate in the metabolism can be followed. The types of tracers used to investigate lipid metabolism are so far: a) characteristic fatty acids, b) fats containing deuterium, and c) radioactive phosphorus (for the study of phospholipid metabolism).

*Sinclair* (1929) fed fats of high iodine value (cod liver oil) and was able to follow to some extent the way of the fat by the change in iodine number of the lipids. The method gives only a rough idea of the processes, and since the liver is able to saturate and desaturate fatty acids, the method can never become reliable. On the other hand it is well known that large amounts of the fatty acids from the food pass unchanged into the fat depots, and that characteristic fats from the food may change the body fat to a surprising degree.

Later *Sinclair* (1936 a. o.) used elaidic acid as tracer in similar work. Iodized fatty acids have been used as indicator by *Artom* (1933). The eleostearic acid used by *Miller & Burr* (1937) may be spectroscopically distinguished, but is not well tolerated by the animals. Later *Miller & al.* (1939) found a method to conjugate the double bonds of linoleic acid, and prepared a fat which could be spectroscopically distinguished and quantitatively determined in body fat.

*Schoenheimer & al.* have made excellent work on the metabolism of fats, using fatty acids with heavy hydrogen built into the molecules in known position. The investigations have thrown much light upon the intermediary metabolism of fat, but unfortunately the method seems not to be well suited for study of the mechanism of fat transport through the intestinal wall.

The radioactive isotope of phosphorus ( $^{32}\text{P}$ ) has been used for the study of phospholipid metabolism (e. g. *Hevesy & Lundsgaard* 1937, *Fries & al.* 1938). Since the  $^{32}\text{P}$  is introduced as inorganic phosphate, it gives primarily evidence of the synthesis of phospholipid molecules only. It is not possible to follow the catabolism in the fatty acid part of the phospholipid molecules by means of this indicator.

### The chemical processes in fat absorption.

The chemical processes in the epithelial cells have been much discussed, but there has been little experimental work made that can contribute to the solution of the problems.

The theory of a synthesis of phospholipids during absorption and transport of fats has gained much favour.

The first experiments on this matter were made by *Bloor* (1915, 1916). He found, during fat absorption in dogs, an increase in the lecithin content in blood. A more detailed examination showed an increase in the total fatty acids both in the plasma and in the corpuscles, and further a large increase in the lecithin content of the corpuscles but little in plasma. There were no definite changes in cholesterol.

*Bloor* concluded: a) Blood corpuscles (red or white) take up fat from the plasma and transfer it to lecithin, b) most or all fat is so transformed, c) lecithin is an intermediate step in fat metabolism (*Bloor* 1916). These conclusions seem to have been too extensive.

The rise in lecithin content in blood has been confirmed by many authors. *Süllmann & Wilbrandt* (1934) also found an increase in the phospholipid content of the lymph flowing from the intestine during fat absorption (rabbit).

As mentioned above *Sinclair* (1929, 1932) followed changes in the fatty acid fraction of phospholipids by feeding characterized fatty acids as tracers. He tried to find support for the theory that the phospholipids of the intestine might be an intermediate stage in the resynthesis of fat from its absorbed constituents. If this is true, the fatty acids of the phospholipids in the mucosa should assume the characteristics of the fat which is absorbed.

During the absorption of highly unsaturated fat, *Sinclair* (1929) found a large increase in the unsaturation of the phospholipid fatty acids, but no increase in the phospholipid amount in intestinal mucosa and liver. In intestinal (smooth) or skeletal (striated) muscle there was no change, neither in unsaturation nor in amount. He

concluded: "It is suggested that absorbed fatty acids are transformed into phospholipid within the intestinal mucosa as an essential step in the resynthesis of neutral fat." If small amounts of unsaturated fat were fed, a marked increase in the level of unsaturation of the liver phospholipids was produced, but it had no apparent effect on the degree of unsaturation of the neutral fat.

Later *Sinclair* (1936) used elaidic acid as a tracer. In cats fed elaidic acid for several days as high as 37 per cent of the plasma phospholipid fatty acids were elaidic acid. No elaidic acid could be detected in the red corpuscles, i. e. the red corpuscles do not participate in phospholipid transport. This also means that *Bloors* theory of the rôle of the red corpuscles in transport of fat as phospholipids must be given up.

*Barnes & al.* (1941, 1) found no parallelism between the rate of incorporation of corn oil fatty acids into mucosa phospholipids and fat transport. They found the phosphorylation theory somewhat doubtful, and concluded that the slowness of the entry of the fatty acids into the phospholipids and the minute amounts present during fat absorption indicate that phosphorylation plays no essential part in absorption.

*Reiser* (1942) found no change in the phospholipid content of the duodenal mucosa during fat absorption in swine.

### Radioactive phosphorus as indicator.

The experiments which try to follow the metabolism of phospholipids using radioactive phosphorus as indicator show that the phosphorus of the lipids in the intestine and the liver is exchanged at a much higher rate than in other organs.

*Hevesy and Lundsgaard* (1937) found only a moderate increase in labelled P in the blood phospholipids after administration together with oil and, although the increase exceeded that found when  $^{32}\text{P}$  was given alone, they came to the conclusion that lecithin was formed outside the intestinal tract. The phospholipids of the intestine were not examined.

*Fries & al.* (1938) found that, among the organs, the small intestine plays the major part in phospholipid turnover in absence as well as in presence of ingested fat. A larger part of the  $^{32}\text{P}$  was found in the phospholipids of the small intestine when olive oil was given simultaneously. Unfortunately it is impossible from the values given by *Fries* to see if the rise in activity is due to an increased

amount of lecithin in the intestine during fat absorption, and it is also impossible to calculate how much of the phospholipid has been submitted to metabolic changes.

### The phosphorylation theory.

*Verzar* and his collaborators have done much experimental work in order to demonstrate the importance of the phosphorylation for absorption and metabolism of fats.

*Verzar & Laszt* (1934, 1) found a more rapid absorption of oleic acid when given together with glycerophosphate or glycerol and phosphate. Phosphate or glycerol alone had no influence. They concluded that a phosphorylation in the mucosa takes place during absorption of fatty acids.

The experiments were made on anesthetized rats, from which 50 cm of the intestine were tied off. After washing the isolated loop the fat was injected into it. The absorption was determined by analysis of the contents. The work gives no certainty that the blood circulation has been kept adequate and that the loops have functioned normally in the experiments. The increased absorbing function may very well be due to a better function when combustion material for the absorption work is supplied in the form of glycerophosphate.

Further, the situation has some points similar to the increase in glucose absorption found when sodium phosphate is given together with it (*Magee & Reid* 1931). Their observation led to a conclusion of phosphorylation in the absorption process (which is probably correct). *Laszt* (1935) however showed that the observation could not be taken as a proof for a phosphorylation because the stimulation was due to the pH, — borate or acetate buffers gave the same increase.

*Verzar & Laszt* (1934, 2 & 1935, 1) used poisoning with mono-iodoacetic acid and phlorrhizin to demonstrate the necessity of a phosphorylation of the fatty acids in the course of the resynthesis to neutral fat during absorption.

Subcutaneous injections of iodoacetic acid prevented fat absorption, but since the general condition of animals poisoned with iodoacetic acid is always poor and the normal glucose metabolism is put out of function, it is difficult to regard the results as a proof for the theory. The inhibition of the uptake found by iodoacetic acid poisoning may as well be due to the failing power of the cells to

supply energy for absorption work as to a depression of the hypothetical phosphorylation of the fatty acids.

*Sinclair* (1941) fed elaidic acid to rats poisoned with iodoacetic acid. He found no difference in the phospholipid content of the intestine nor in the mixture of their fatty acids as compared with non poisoned animals.

*Verzars* experiments with phlorrhizin gave also a retarded absorption, but the doses necessary were 50 to 100 times as large as those needed for causing an extreme diabetes. (A better method for poisoning the intestine is to introduce the substances directly into the intestine as used e. g. by *Carlsen* (1945). In this way the general intoxication is of less strength or totally prevented).

In my opinion the result of phlorrhizin poisoning speaks against the theory of a phosphorylation process that is directly inhibited by the poisons used. It is also difficult to see why a substance of a chemical constitution so different from glucose should take part in synthetic processes of a character similar to the phosphorylation of carbohydrates. On the other hand the experiments give no evidence against the theory of a phospholipid formation.

The theory of phosphorylation was also the reason for *Verzars* investigations of the rôle of the adrenal cortex hormone in fat absorption (*Verzar & Laszt* 1935, 2—3). The absorption of fat was greatly inhibited by adrenalectomy, and it was decisively demonstrated that the cortex hormone restored the ability of fat uptake.

The poor fat uptake after iodoacetic acid poisoning or adrenalectomy was followed histologically by *Jeker* (1935). Against the normal picture with abundant fat in the epithelial cells and the lymph he found no fat in the poisoned or adrenalectomized animals. In the latter, the normal conditions could be re-established by the administration of cortex hormone.

Later on *Jeker* (1936) used a staining method (*Fischler's* staining) which according to him should with certainty distinguish between neutral fat and fatty acids. *Jeker* was aware of the fact that the method had been strongly attacked (*Jeker* 1936, p. 3), but he gave no new evidence in support of the method. The very nice results must be seen from this point of view. During fat absorption in normal animals he found that at first a *Fischler* positive substance (fatty acids) appeared, but later a synthesis to neutral fat begun, the *Fischler* positive substance disappeared and instead of it a sudan positive substance (neutral fat) was found.

During iodoacetic acid poisoning only *Fischler* positive substance was found, indicating that the synthesis of neutral fat (primarily the phosphorylation of the fatty acids) was inhibited. *Verzar & Jeker* (1936) found the same histological picture by adrenalectomy, and normal conditions were restored by cortex hormone.

Unfortunately the work of *Barnes & al.* (1939—1941) showed that the findings were probably due to the poor condition of the intestine of adrenalectomized rats. If the condition of the animals was maintained by giving them 1 per cent sodium chloride for drinking instead of water, the absorption rate of corn oil fatty acids or their methyl esters was not affected by adrenalectomy (*Barnes & al.* 1939).

*Bavetta & al.* (1941) supported the experimental work of *Verzar*, and found inhibited fat absorption by adrenalectomy, even if the animals were maintained with salt solutions. They were restored by cortex hormone. In 1943 they found the phenomenon restricted to the long chain fatty acids, while short chain acids were absorbed at normal rate after adrenalectomy.

*Barnes & al.* repeated and confirmed their experiments (1941, 2). They express the opinion that they have brought no evidence against adrenal hormonal regulation of fat transport, but that the adrenals are not necessary for a) the normal rate of fat absorption and b) the normal rate of incorporation of absorbed fatty acids into the intestinal mucosal phospholipids and neutral fat. They conclude that: "It is quite clear that if the rate of accumulation of new fatty acids is a criterion of rate of phosphorylation, the cortical hormone plays no direct part in fatty acid phosphorylation."

On the basis of the published data it seems difficult to decide whether the opposite results are due to a failure in the salt maintenance of *Bavettas* animals or an experimental failure in the work of *Barnes*. (That the adrenals have some effect upon fat metabolism was shown also by *Barnes & al.* (1941, 3), who found that adrenalectomized rats maintained in good condition have impaired ability to deposit fat in the neutral fat stores of the liver. Extract of adrenal cortex restored the ability. No such impairment was noticed in the transport of the acids into liver phospholipids).

In the present work it was attempted to decide how large a fraction of the fatty acids is present as soaps at the pH of the intestine, and to investigate the presumed phosphorylation process during absorption.

## TECHNIQUE

### EXTRACTION TECHNIQUE

In physiological and biochemical investigations, lipids to be investigated are commonly isolated from the tissue by extraction.

A total extraction can be carried out either by repeated extractions, or in an apparatus which gives a constant stream of the pure solvent (*Soxhlets* method).

From the solution obtained the lipids are mostly isolated by evaporation of the solvent. If the constitution of the fat (e. g. double bonds, free fatty acids etc.) is of interest the evaporation is carried out in a carbon dioxide or nitrogen atmosphere.

The tissue must be dehydrated before the extraction. This may take place by the addition of large amounts of neutral salts (e. g. sodium sulphate) but mostly an evaporation of the water is used. Freezing of the tissue, pulverization and drying in vacuo is regarded as a very lenient method.

It has turned out that the total amount of lipids is not removed from the tissue by extraction. Even if different solvents such as acetone, alcohol, ether, petrol etc. are used, and even if the tissue is extremely finely pulverized, there remains a fraction of the lipids.

This remnant can be liberated if the tissue is destroyed, either by pepsin digestion or destruction by boiling with potassium hydroxide. In the latter case the fats are also saponified, and the free fatty acids can then be extracted after acidifying the mixture.

The common opinion is that the non extractible lipids are combined with the proteins of the tissue and that they are a structural element of the protoplasma. This fat quantity is usually only a minor part of the total.

The alkali destruction of the tissues cannot be used in investigations of phospholipids, because these are totally saponified by this treatment.



## Extraction of small tissue samples.

It is very difficult to handle small quantities of fat solvents or lipids. Most of the fat solvents used are easily volatile, and when a lipid solution is evaporated the lipid has a great tendency to "creep" along the surfaces of the vessels used. If, e. g., a single drop of an ether solution of any fat is placed in the middle of a watchglass of five cm diameter the fat will, when the ether has evaporated, be found over almost the whole surface of the glass, and the major part will even be found near the edge of it.

These difficulties can be almost completely overcome when a known volume of solvent is used for extraction and aliquot parts of it are pipetted off for analysis. This principle is developed in the micromethods for fat analysis described by the writer (1942, 1944) and gives generally a simple and safe method for handling very small lipid samples.\*)

Because the solvent used in the extractions of the intestine samples is very volatile, the extractions were carried out in closed glass ampoules.

The extraction of a sample was carried out as follows:

The sample was dried, weighed and brought into a glass tube of about 80 mm length and 8 mm width. The upper part of the tube was then drawn out in such a way that a constriction of about 2 mm in diameter was formed. Two ml of the solvent (equal parts of absolute alcohol and ethyl ether) were pipetted down into the ampoule, which was then sealed by drawing off the upper part in a gas burner.

Now the extraction could take place without disturbance from evaporation, and the ampoules might be stored any desired length of time before further treatment. Because the tissue samples were very thin, the lipids were soon dissolved and evenly distributed in the solvent, and apart from the non extractible lipids the extraction was complete.

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\*) The "creeping" of the lipids has been found not to disturb in the following special cases: When the small sample (5—10  $\mu$ l) used for fatty acid determination is pipetted into the small ampoule used in the microanalysis, the solvent may be evaporated in vacuo without the fat creeping to any harmful extent. When the fatty acids for the final titration are pipetted from the ampoule into the special titration vessel, the solvent may also be evaporated in vacuo. The fatty acids do not "creep" above the level reached by the titration medium later added.

In most cases the analyses of this investigation were carried out the day after the ampoules were filled. The tissue samples were from 10 to 100 mg in size. The lipid radioactivity of such samples is, when moderate doses of  $^{32}\text{P}$  are administered, relatively low. Therefore it was desired to use the major fraction of the samples for activity countings. The methods used for chemical analyses favoured this object to a great extent, as they only claimed less than 1/10 part of the total amounts present. For phosphorus determinations 200  $\mu\text{l}$  were taken out, for fatty acid determination only 5—10  $\mu\text{l}$ . Because in work of this character the variations in different experiments and even in different samples from one animal are very large, it is very fortunate that the different analyses can be carried out on one and the same sample.

Is the extraction method adequate?

The extraction technique used is so simple that its sufficiency can be questioned.

It is very easy to control if the extraction of phospholipids is complete and quantitative by means of tissue samples where the phospholipids contain radioactive phosphorus. If the radioactivity is relatively high even minute quantities of phospholipids can easily be detected and quantitatively determined by counting of the radioactivity.

When samples from the intestine had been extracted in the usual manner, they were brought into another ampoule and extracted once more. It was not possible to detect radioactivity in this new extract, i. e. the quantities were less than the sensitivity of the counting technique.

Of course some traces of phospholipids remain in the solvent with which the tissue sample is soaked. By the calculations for the sensitivity of the activity determinations, it was with certainty demonstrated that less than 2 per cent of the total phospholipid amounts were left in the tissue after the first extraction. This small fraction is of no importance as compared with the other errors of the determinations. Of all the quantitative determinations carried out, the phosphate analyses of the phospholipids are the least accurate, their analytical error being probably not below 5 per cent. In relation to this, there is no reason to aim at an accuracy below a few per cent in any of the other analyses.

## THE FATTY ACID TITRATION

The fatty acids were determined according to the method previously described by the writer (1942). The technical procedure has been somewhat simplified, and needs not so much care and attention during all the operations as before.

The process is simply: In a small glass ampoule the fat is saponified with alcoholic sodium hydroxide and a known volume of toluene. Then a surplus of hydrochloric acid is added. The fatty acids are taken up by the toluene, and an aliquot part of this is pipetted off for titration of the acids.

If the toluene and water phases shall separate readily, the alcohol content must not be too large. About 10 per cent of alcohol was found to be harmless.

When analyses were carried out on lipids from the intestine, difficulties in separating the toluene and water phases appeared. Therefore, after saponification, the ampoule was opened and all the toluene and alcohol were evaporated off. Then the hydrochloric acid was added, and at last the known volume of toluene. Because all the alcohol was removed, a larger amount of alcoholic potassium could be used for the saponification, the advantage of this was an easier pipetting technique and a more reliable saponification. But the most remarkable advantage was that after the pipetting of the known volume of toluene, the ampoule was not opened again before the final pipetting off of the aliquot part. — In the earlier manner of proceeding the toluene was added first and the ampoule was reopened for adding HCl, a method which required much more attention and rapidity in the work. The new procedure involves less possibility of error, is more convenient and only the last sealing of the ampoule needs be carried out with special attention.

## THE PHOSPHATE DETERMINATION

The phospholipids were quantitatively determined by an analysis of their phosphorus content.

The phospholipids were destroyed with sulfuric acid and the phosphorus determined by a modification of the Fiske-Subbarow principle (see *Kjerulf-Jensen*, 1942 p. 83).

From the alcohol-ether extract 0.2 ml were pipetted off into a test-tube of resistant glass. Then the solvent was evaporated on a steam bath.

The common method for destruction of the phospholipids is to

heat them with a mixture of sulfuric acid and nitric acid. Before the analysis is carried further, every trace of nitric acid must be removed. It appeared that this is not easy to carry out in large series of analyses, in many of the samples traces of oxydating compounds had remained.

It appeared that a small quantity of sulfuric acid (0.1 ml was used) and a few drops of hydrogen peroxide was an excellent mixture for destruction of phospholipids. By heating the samples with that mixture, a rapid development of gases appeared. If the mixture became darkly coloured by further heating, more peroxide was added. The process was repeated until no dark colour appeared by heating the samples.

The surplus of peroxide now present was removed by placing the test-tubes in an oven at  $150^{\circ}\text{C}$  for about  $\frac{1}{2}$  hour. If traces of organic matter were still present the samples attained a dark colour again. Now a single drop of peroxide and a renewed stay in the oven completed the process.

The sulfuric acid must be carefully neutralized before the analysis is carried out. The colorimetric determination of phosphorus is performed in strongly acid solution (1 n sulfuric acid) but the strength of the colour is sensitive to even small variations in the acidity of the solution. Therefore it is best to remove the surplus of sulfuric acid remaining from the destruction process before the reagents are added.

The samples are diluted with 5 ml of water. The neutralization is then carried out with strong sodium hydroxide (10 per cent) against phenolphthalein as indicator. Care must be taken not to add too large a surplus of sodium hydroxide, because the colour of phenolphthalein rapidly disappears in strongly alkaline solution. Afterwards the samples are brought back to the acid range of the indicator by dropwise addition of dilute hydrochloric acid (1/10 normal).

Now all the samples have to be diluted to the same volume, e. g. 8 ml. A mark on the vessel indicating this volume facilitates the procedure. Then the reagents are added, namely 2 ml of a 2.5 per cent solution of ammonium molybdate in 5 n sulfuric acid and 0.5 ml of a solution of 1.5 g of amidol and 15 g of sodium sulfite in 100 ml of water. (The last solution must be freshly prepared each day).

The colour is measured in an electric photometer after placing

the samples for 20 minutes in a water bath at 40° C or after standing over night at room temperature.

Known quantities of a standard phosphate solution must always be carried through the analytical procedure for calculation of the unknown samples, because the strength of the colour is not absolutely constant from one series of analyses to the next.

## RADIOACTIVITY MEASUREMENTS

### General.

The radioactivity of the phospholipids extracted from the tissue samples was determined by means of a Geiger-Müller counter (Levi, 1941).

The counting tube as well as the amplifying system were of an improved construction as described by *Ambrosen & al.* (1945). The counting device was used in connection with a "scale of four" system (*Ambrosen*, p. 205) through which each fourth of the impulses reached the recorder. At activities up to 1000 impulses per minute each fourth impulse will be recorded practically without losses. At activities of 1500 imp/min each fourth impulse is recorded with a loss of 2 per cent, and at 2000 imp/min the loss will be 5 per cent. At higher activities such corrections are not accurate since the recording device cannot bear a much higher strain.

The accuracy of the measurements is larger when a larger number of impulses is registered. Approximately the probable fluctuation of the single determination may be calculated in the following way:

The statistical fluctuation is proportional to the square root of the number of disintegrations of the radioactive substance. The number of impulses registered by the counter,  $N_{reg}$ , is proportional to the number of disintegrations,  $N$ . Hence we have the relative uncertainty of the single determination,  $\sigma$ , by the following formula:

$$\sigma = \frac{\sqrt{N_{reg}}}{N_{reg}} \quad \text{or} \quad \sigma = \frac{1}{\sqrt{N_{reg}}}$$

(As mentioned the formula is only valid when  $N_{reg}$  is proportional to  $N$ , i. e. when the register records the impulses from the Geiger-Müller tube practically without losses).

If 10 000 impulses have been registered, we have an uncertainty or probable fluctuation of:

$$\sigma = \frac{1}{\sqrt{10\,000}} = \frac{1}{100} \quad \text{or} \quad 1 \text{ per cent}$$

If 2500 impulses are registered, we have:

$$\sigma = \frac{1}{\sqrt{2500}} = \frac{1}{50} \text{ or } 2 \text{ per cent}$$

If 400 impulses are registered, we have:

$$\sigma = \frac{1}{\sqrt{400}} = \frac{1}{20} \text{ or } 5 \text{ per cent}$$

In the present work the errors arising from other sources (isolation of the lipids, phosphate analysis etc) were considerable and probably of an order of magnitude of not less than 5 per cent. Therefore it was assumed that counting of 400 impulses, giving a probable fluctuation of  $\pm 5$  per cent, was sufficient in all the experiments carried out on animals.

The half life period of radioactive phosphorus is 14.3 days. From one day to the next the activity of a preparation decreases 4.8 per cent. Therefore, when different preparations are not measured the same day their activity cannot be directly compared.

The activity of radioactive substances decreases following a logarithmic curve given by the formula:

$$y_t = y_0 \cdot e^{-\lambda t}$$

where  $y_0$  and  $y_t$  are the original activity and the activity at the time  $t$  respectively.  $\lambda$  is a constant characteristic for each radioactive substance, and means the per cent fraction of the substance which disintegrates in the time unit.

Instead of this we may use the half life period of the substance, which gives the formula:

$$a_m = a_0 \cdot 0.5^m$$

where  $a_m$  is the activity after  $m$  half life periods and  $a_0$  the original activity.

If we wish to calculate the activity after a given number of days, we get the formula:

$$a_n = a_0 \cdot 0.5^{\frac{n}{p}}$$

where  $a_n$  is the activity after  $n$  days and  $p$  the half life period given in days.

Hence, if on the  $n$ -th day we have observed an activity,  $a_n$ , of a

preparation of  $^{32}\text{P}$  (half life period  $p = 14.3$  days) we have the original activity:

$$a_n = a_o \cdot 0.5^{\frac{n}{14.3}}$$

or

$$a_o = \frac{a_n}{0.5^{\frac{n}{14.3}}}$$

When many preparations are measured this recalculation as to the same point of time is very inconvenient, and another procedure is usually adopted.

One of the samples of the radioactive substance is chosen as a standard and the strengths of the other preparations are calculated in relation to this one. The standard is always counted in connection with a series of unknown samples, their strengths are then calculated in per cent of the standard.

E. g. if we have a standard preparation that gives 100 imp/min and another sample  $a$  that gives 25, the strength of the sample  $a$  is 25. If on another day, the activity of the standard has decreased to only 80 imp/min, and a sample  $b$  gives 20 imp/min, the activity of  $b$  is calculated to be 25. In this way it is immediately found that the samples  $a$  and  $b$  have the same activity, they may be compared directly without any further correction even if they are not measured at the same time.

In the case of  $^{32}\text{P}$  the activity at any time is 95.2 per cent of the activity 24 hours earlier, i. e. about 5 per cent of the activity is lost in one day and night. Therefore the error produced by comparing preparations measured on one and the same day needs not be corrected for when an accuracy of a few per cent is sufficient.

### Phospholipids.

The preparations to be measured are placed in small aluminium dishes 12 mm in diameter and 2 mm deep. It is practically impossible to place lipid substances on a limited area and equally distributed over it. All lipid substances tend to spread along surfaces, and especially when a solution of the lipid is evaporated the lipid substance has a great tendency to "creep" (page 26).

If a single drop of a fat solution is placed in the bottom of the aluminium dish, the fat will have gathered along the ridge when

the solvent has evaporated. An even distribution of it is of importance to a correct counting of the activity. Therefore the solution of the lipids was evaporated on a small disk of filter-paper, 10 mm in diameter, which was placed in the aluminium dish.

The aluminium dish was placed on a heated copper block, and the solution of lipids pipetted directly down on the filterpaper. In this way the solvent evaporates immediately, and the lipid stays distributed in the filter-paper. To control that no part of the lipid has spread outside the paper disk, the disk is moistened with a drop of sudan solution. Then all the fat is stained intensively red, and if any part of it comes outside the paper it will immediately be detected. Without staining it is impossible to see small fat droplets on the aluminum dish. If the fat is stained, it will as a rule even be possible to suck it up into the filterpaper again.

It is of importance that the filter-paper does not absorb perceptible quantities of the radiation. This is easily proved by counting a preparation containing  $^{32}\text{P}$ , and then cover it by a disk of filter-paper and counting it again.

In table 1 is given an example. The absorption in the filter-

TABLE 1.

*The absorption of the radiation from  $^{32}\text{P}$  in filter-paper and in fat.*

	imp/min	Absorption, per cent
Sample of $^{32}\text{P}$ .....	406.8	
Same, covered with filter-paper .....	402.0	1.2
Same, the filter-paper soaked with 10 mg of fat .....	391.6	3.8

12 000 impulses counted. Probable fluctuation  $\pm 0.9$  per cent.

paper is of the order of magnitude of 1 per cent, which is of no importance to the measurements of the phospholipids. Further we may suppose that when the  $^{32}\text{P}$  is distributed in the filter-paper the absorption is less than when all the radiation must pass the paper disk.

It might be assumed that the lipid substances which contain the  $^{32}\text{P}$  and also those which are extracted together with them absorb some of the radiation from the  $^{32}\text{P}$  built into the lipid molecules. The substances contain only light atoms, C, H, O, N and P, and therefore the absorption cannot be expected to be of considerable magnitude. A simple control experiment is easily carried out. The



preparations of phospholipids were only a few milligrams, and almost never above 5 mg. A sample of  $^{32}\text{P}$  was covered with a disk of filter-paper soaked with 10 mg of rats fat. The absorption in this preparation must be several times larger than in any of the phospholipid preparations because all the radiation must pass through the total distance of absorbing material. It was found to be less than 4 per cent (table 1) and consequently the absorption in the phospholipid preparations must be of no importance. Since the errors in different samples will be of the same order of magnitude, corrections for these minute errors were found unnecessary.

When  $^{32}\text{P}$  is used as an indicator of phospholipids it is of great importance that the solvent used for extraction of the lipids does not dissolve even traces of other phosphorus containing substances. Especially in experiments of short duration the free phosphorus is highly active in relation to the phosphorus of the lipids, and quantities which cannot be detected by chemical analysis can completely disturb the measurements.

Dr. G. Hevesy has informed me that the following procedure is sufficient for testing if "free" phosphates are present in the solution.

To the alcohol-ether extract is added very well dried and finely pulverized sodium phosphate. If free phosphates are present in the solution, they will exchange with the phosphates added. If the addition has no effect upon the lipid radioactivity, no appreciable amounts of non-lipid phosphate have been dissolved.

In those cases in which I treated the extracts of rats intestine in this way the activity was not altered by the treatment. But the absolute strength of these preparations was almost so low (10—20 imp/min) that the probable fluctuation of the countings could not with reasonable efforts be brought down to below about  $\pm 5$  per cent. Therefore relatively small changes in the activity might have taken place without being detected.

From some animals used in experiments with  $^{32}\text{P}$  the livers were taken out, dried, finely pulverized and extracted with the alcohol-ether mixture. In such animals the free phosphorus has an extremely high activity, but in the liver considerable amounts of active phosphatides are already present.

One part of such an extract was treated with sodium phosphate in the manner described, and equal volumes of the treated and untreated extracts were taken out for counting (table 2).

TABLE 2.

*Radioactivity in 0.2 ml of an alcohol-ether solution of rats liver. Treated with pulverized sodium phosphate. Activities given in per cent of untreated sample.*

Duration of treatment	10 min	1½ hours
Not treated with Na-phosphate .....	100	100
Treated with Na-phosphate .....	99	102

Probable fluctuation of single determination:  $\pm 2$  per cent.

It is evident that no perceptible amounts of activity disappear during this treatment.

The experiment was repeated with another extract which was treated with sodium-phosphate for a longer period (table 3).

TABLE 3.

*Radioactivity in alcohol-ether extract of rats liver. Treated with sodium phosphate for 2 days. Activities given in per cent of untreated sample.*

Untreated sample .....	100.0
Treated with Na-phosphate .....	100.4

Probable fluctuation of single determination:  $\pm 1$  per cent.

## EXPERIMENTAL PART

### THE POSSIBILITY FOR ABSORPTION OF SOAPS IN THE INTESTINE

#### THE CHEMICAL CONDITIONS FOR THE PRESENCE OF SOAPS IN THE INTESTINAL CONTENT

During the many discussions of theories concerning fat absorption in the intestine, many suppositions have been made which are not in accordance with the chemical facts.

With great power *Pflüger* defended the theory that, after the fats have been split up by the lipase, the fatty acids are converted to soaps by means of the sodium carbonate of the pancreatic juice. His chief idea was that, since carbohydrates and proteins are hydrolysed and taken up in the water soluble form, also the fats ought to be taken up as water soluble compounds, i.e. as soaps.

Later on it has appeared that the reaction of the small intestine is actually not alkaline, such as was earlier supposed. The reaction is usually slightly acid, in different animals and different parts of the small intestine varying between pH 5.5 and 7.

If an attempt is made to neutralize a watery solution of soaps, it will be found that the soaps split up and that the fatty acids separate out from the solution. This well known fact led to the idea expressed by most authors, namely: "Soaps cannot exist below pH = 9" or "Soaps cannot exist at neutral reaction."

Such assertions cannot be accepted from a chemical point of view. If acid is added to a soap solution there will, irrespective of any surplus of acid, still be a certain amount of soap present in the solution. The proportion may be calculated from the equation of mass action, supposing that the dissociation constant ( $K_a$ ) of the acid and the activities of the components in the solution are known.

The first attempt of the writer to accomplish such a calculation for the fatty acids at the pH of the intestinal content was hindered by the total absence of determinations of the  $K_a$  of higher fatty acids. This is surely due to the very low solubility of the acids, and a further difficulty is that the watery solutions of their salts are not simple molecular-disperse systems.

#### DISSOCIATION CONSTANTS OF FATTY ACIDS

The dissociation constants of short chain fatty acids have been determined, and in table 4 the constants to be found in the literature are collected. The determinations made by different authors differ somewhat, according to different methods etc., but usually not more than  $0.1 \cdot 10^{-5}$ .

TABLE 4.

*The dissociation constants,  $K_a$ , of straight chain fatty acids of from 1 to 8 carbon atoms.*

	Carbon atoms	$K_a$	Temperature
Formic acid .....	1	$21.4 \cdot 10^{-5}$	25° C
Acetic acid .....	2	$1.86 \cdot 10^{-5}$	25° C
Propionic acid .....	3	$1.45 \cdot 10^{-5}$	25° C
Butyric acid .....	4	$1.48 \cdot 10^{-5}$	25° C
Valeric acid .....	5	$1.51 \cdot 10^{-5}$	18° C
Hexoic acid .....	6	$1.44 \cdot 10^{-5}$	25° C
Heptoic acid .....	7	$1.42 \cdot 10^{-5}$	18° C
Octoic acid .....	8	$1.44 \cdot 10^{-5}$	25° C

It will be seen that, apart from the two lowest links, there is a striking constancy in the dissociation constants of the fatty acids. Chains of from 3 to 8 carbon atoms give no variations in the  $K_a$ . From a physical point of view, a further addition of methylen groups ( $-\text{CH}_2-$ ), i. e. an increase in the length of the paraffin chain cannot be expected to give any change in the  $K_a$ . Therefore we may suppose that the  $K_a$  of the higher fatty acids is also of the same size ( $K_a = 1.4 \cdot 10^{-5}$ ).

The bile acids have chemical properties which are in many respects similar to those of fatty acids. From theoretical considerations *Kumler & Halverstadt* (1941) predicted that many bile acids should have dissociation constants almost identical with those of fatty acids, i. e.  $K_a = 1.0 \cdot 10^{-5} - 1.4 \cdot 10^{-5}$ . Earlier determinations (e. g.

Josephson 1933) showed that bile acids are very weak. Then Kumler & Halverstadt measured the  $K_a$  in alcohol-water mixtures and from this the constants in water were calculated. They appeared to be completely in accordance with the predictions. Examples of the results are given in table 5.

TABLE 5.  
*Dissociation constants of bile acids.*

	Kumler & Halverstadt	Josephson
Desoxycholic acid .....	$1.08 \cdot 10^{-5}$	$3.8 \cdot 10^{-7}$
Cholic acid .....	$1.12 \cdot 10^{-5}$	$6.46 \cdot 10^{-6}$

The fatty acid salts as well as the bile acid salts do not form simple molecular solutions but are "colloidal electrolytes" which form "ionic micelles" in watery solutions (Roepke & Mason, 1940), and this is probably the explanation of their apparently irregular behaviour.

By a lucky chance I received the information that Mr. A. Nærland, C. E., The Technical University of Norway, has measured the dissociation constants of fatty acids in alcohol. He carried out the measurements in 95 per cent alcohol. From hexoic acid ( $C_6$ ) to hexadecanoic acid ( $C_{16}$ ) he found a variation in the  $pK_a$  of less than 0.01 ( $\Delta pK_a < 0.01$ ). These data are unpublished, and given me as personal communication by Mr. Nærland, to whom I owe the heartiest thanks for the permission to publish them. This investigation gives the experimental evidence for the correctness of the conclusion that the length of the hydrocarbon chain has no influence upon the  $K_a$  of the fatty acids of a size larger than  $C_3$ .

Now we can take up again and discuss the assertion that "soaps cannot exist at the pH of the intestine."

Disregarding the consequences of the low solubility of the acids, we may calculate for the fraction present in solution that if

$$K_a = 1.4 \cdot 10^{-5}$$

there will be equal parts of soap and fatty acid in the solution at  
 $pH = 4.85$

From this it follows that at the pH of the intestine the major fraction of the fatty acids in solution is present as soaps.

## EXPERIMENTAL

To what extent these theoretical reflections are valid in the actual chemical systems of the intestine may to some extent be seen from electrometric titration curves of the fatty acids.

The solubility of the sodium salts of stearic or palmitic acid at room temperature was found to be too low for using them in the experiments. However sodium oleate was easily soluble in water, and the electrometric titrations were carried out on such solutions. (The position of the double bond in oleic acid is so far from the carboxylic group that no influence upon the dissociation can be expected.)

The use of hydrogen electrodes in soap solutions is probably very inconvenient, and therefore the electrometric measurements were carried out by means of glass electrodes. These electrodes have a systematic error in strong alkaline solutions, above  $\text{pH} = 9$ , but the titration curves for pure NaOH solutions did not differ to any perceptible extent from the theoretical values, and therefore the error of the electrode used by the writer is of no importance to the present investigation.

The titrations were carried out on 1/100 normal solutions. In 50 ml water, 0.5 mE of the fatty acid + 1 mE NaOH were dissolved. The solutions were kept  $\text{CO}_2$ -free,  $\text{CO}_2$  from the atmosphere was excluded, and the titration was carried out with  $\text{CO}_2$ -free 1 normal HCl from a burette permitting readings of  $1 \mu\text{l}$ .

## Titration curves of fatty acids.

A titration curve for propionic acid ( $\text{C}_3$ ,  $K_a = 1.45 \cdot 10^{-5}$ ) is given in fig. 1. The curve corresponds exactly to the theoretical titration curve for an easily soluble acid of this strength. First the surplus of NaOH (0.5 mE) is neutralized, then on a minute addition of HCl the reaction changes from  $\text{pH} 10$  to  $\text{pH} 7$ , and from this point the titration of the weak acid begins. In every point the values observed for the  $\text{pH}$  are the same as those calculated.

The titration curve for oleic acid is given in fig. 2.

The surplus of NaOH is neutralized when 0.5 ml of HCl have been added, and further half the quantity of soap is neutralized when 7.5 ml of HCl have been added in all. At this point we find from the curve that the  $\text{pH}$  is about 8. Since half the soap is neutralized the solution contains equal parts of fatty acid and salt, i. e. it looks as if we had an acid with  $\text{p}K_a$  about 8 and  $K_a$  about  $10^{-8}$ .

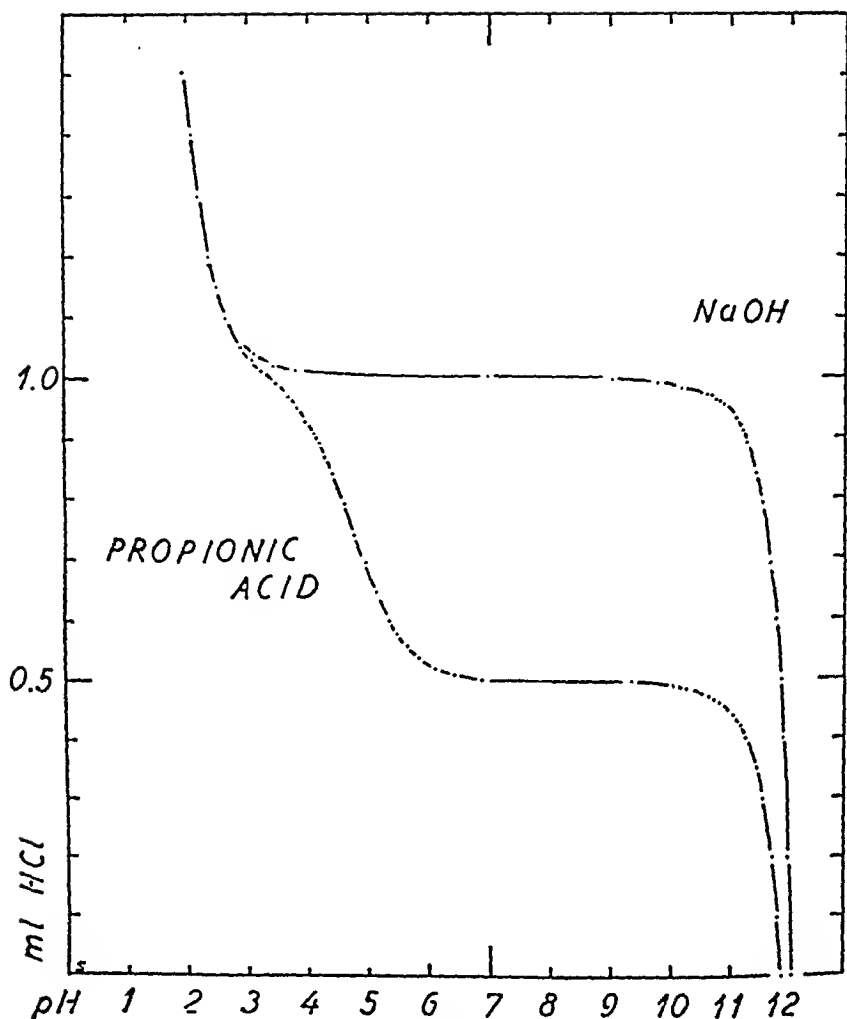


FIG. 1.

Electrometric titration of 0.5 mE of propionic acid with 1 mE NaOH added. Titration fluid 1 n HCl. This curve as well as the curve for pure NaOH, corresponds in every point to the theoretical curve.

Consequently, from this point of the titration curve we find an apparent dissociation constant  $K'_2$  \*) of  $10^{-8}$  against the expected

\*)  $K'_1$  = dissociation constant calculated from the stoichiometric proportions in the reaction system.

$K_2$  = dissociation constant of active acid, supposed to be identical with the true dissociation constant of the acid.

$C'_{HA}$  = the concentration of fatty acid as given by the stoichiometric proportions.

$C_{HA}$  = the active concentration of fatty acid, supposed to be present in molecular dispersed form.

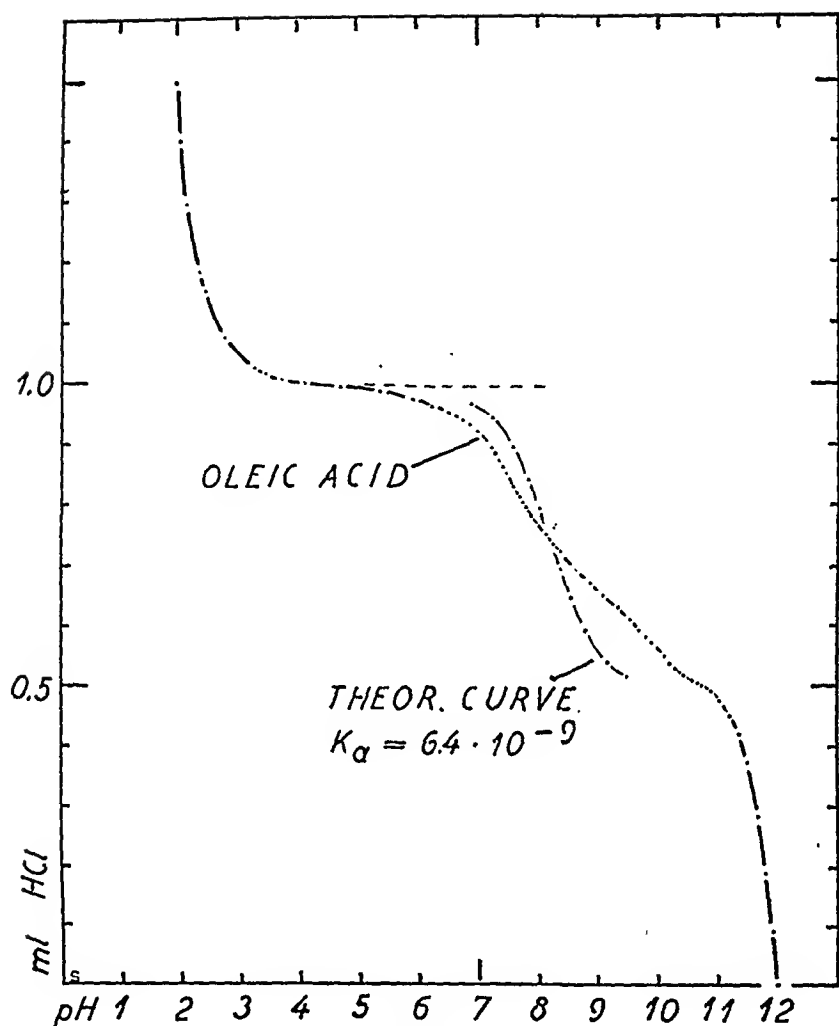


FIG. 2.

Electrometric titration of 0.5 mE of oleic acid with 1 mE NaOH added. In the graph a theoretical curve is drawn, calculated for  $K_a = 6.4 \cdot 10^{-9}$ , which corresponds to the apparent  $K_a$  for the half neutralization point of the oleic acid.

value of about  $10^{-5}$ . How can these apparently contradictory results of theoretical considerations and the experimental facts be explained?

The titration curve in fig. 2 may be roughly explained if we regard the fatty acid as a solid phase of very low solubility.

We have the law of mass action:



$$\frac{C_{H^+} \cdot C_{A^-}}{C_{HA}} = K_a$$

or

$$C_{H^+} = \frac{C_{HA}}{C_{A^-}} \cdot K_a$$

The hydrogen ion concentration,  $C_{H^+}$ , is proportional to the concentration of the acid,  $C_{HA}$ , and reversely proportional to the concentration of the salt,  $C_{A^-}$ . If the acid is precipitated, its concentration will be less than corresponding to the stoichiometric amount, and the  $C_{H^+}$  will also be less than expected.

If in fig. 2 we regard the point where half the salt is neutralized, we can calculate the apparent dissociation constant,  $K'_a$ , for this point. The point corresponds to equal parts of acid and salt, i. e.

$$\frac{C'_{HA}}{C_{A^-}} = 1$$

and consequently

$$C_{H^+} = K'_a$$

From the curve we find that pH is 8.2, and consequently  $pK'_a = 8.2$  and  $K'_a = 6.4 \cdot 10^{-9}$ . This constant is obviously not the correct one, because considerable amounts of the acid have separated out from the water phase at this point.

If we presuppose the very simple conditions that the salt is in real solution and dissociated, that a certain part of the acid,  $C_{A^-}$ , is in real molecular solution and dissociated, and further accept the true dissociation constant of the acid as  $K_a = 1.4 \cdot 10^{-5}$ , we get:

$$C_{HA} = \frac{C_{H^+} \cdot C_{A^-}}{K_a} \text{ and } C'_{HA} = \frac{C_{H^+} \cdot C_{A^-}}{K'_a}$$

For the point of half neutralization of the salt, the  $K'_a$ , the dissociation constant calculated from the stoichiometric concentrations of the reaction mixture, was found to be  $K'_a = 6.4 \cdot 10^{-9}$ .  $K_a$ , the true dissociation constant of the acid is  $K_a = 1.4 \cdot 10^{-5}$ . Hence:

$$C_{HA} = \frac{C_{H^+} \cdot C_{A^-}}{1.4 \cdot 10^{-5}} \text{ and } C'_{HA} = \frac{C_{H^+} \cdot C_{A^-}}{6.4 \cdot 10^{-9}}$$

$$\frac{C_{HA}}{C'_{HA}} = \frac{6.4 \cdot 10^{-9}}{1.4 \cdot 10^{-5}} = 4.6 \cdot 10^{-4}$$

$$C_{HA} = C'_{HA} \cdot 4.6 \cdot 10^{-4}$$

From this we may conclude that (at the pH chosen for the calculation) the dissociation constant calculated for the stoichiometric proportions can be explained by a separation of the major part of the fatty acid, and that only about one part in 50 000 of the fatty acid is actually in solution as active acid. This calculation must be regarded as a very rough approximation, which only gives an indication of the possible state.

It is very difficult to imagine how a direct experimental verification should be carried out. The solubility of the fatty acid must be varying with the pH, and at the pH mentioned there are also ample amounts of soap present. Very little is known about the exact condition of such solutions, to what extent the soap influences the solubility of the acid, the activity of the acid etc.

### Chemical constitution of the fatty acid — soap system.

The chemical conditions in soap solutions have been subject to numerous investigations. An excellent and critical review is given by *Hartley* (1936). In the last years the salts of paraffin chain sulfonic acids have been subject to much interest, because of their extensive technical importance e. g. *McBain & al.* (1939), *Wright & al.* (1939).

This work throws much light upon the conditions of paraffin chain compounds in watery solution. Unfortunately the investigations do not deal with solutions containing free fatty acid, in which the conditions must be still more complicated.

In a fatty acid — soap system we probably have the following dissociated and undissociated particles represented:

TABLE 6.  
*Particles in a fatty acid-soap system.*

	Molecular system				Micelles or colloid particles
Charged particles	H <sup>+</sup>	Na <sup>+</sup>	OH <sup>-</sup>	A <sup>-</sup>	(Mic <sub>A<sup>-</sup></sub> ) <sup>n-</sup>
Uncharged particles	H <sub>2</sub> O		HA		(Mic <sub>HA</sub> )
Hypothetical and questionable particles	A <sub>2</sub> <sup>==</sup> HA <sub>2</sub> <sup>-</sup>		NaHA <sub>2</sub> (acid soap)		(Mic <sub>2A<sup>-</sup></sub> ) <sup>n-</sup> (Mic <sub>HA<sub>2</sub><sup>-</sup></sub> ) <sup>n-</sup> (Mic <sub>NaA</sub> ) (Mic <sub>HN<sub>a</sub>A<sub>2</sub></sub> )

Several of the molecules are only partly soluble in molecularly dispersed form, they tend to aggregate (soaps?) and form micelles and colloidal particles (soaps? acid soaps?) and to separate out (fatty acids). The phases are perhaps to some extent soluble in each other, in the colloid particles etc. The system is of so high complexity that it is impossible to make general conclusions regarding its condition.

Some of the facts, however, are of a character suitable for further discussion.

The  $n/100$  solution of sodium oleate is absolutely clearly transparent. From the work of *McBain* it is well known that the soap solutions do not behave as molecular disperse solutions from the osmotic point of view. E. g. the lowering of the vapour pressure is of a magnitude as in colloidal solutions.

That the soap solutions are not simple semicolloidal solutions is demonstrated by the fact that soap solutions are excellent conductors of electricity (*McBain*). *McBain* and many later authors suppose that the soaps form ionic micelles in watery solution. The calculations made by *Linderstrom-Lang* (1926) on the contrary give evidence to support the view that the properties of the solutions are not due to micelle formation but to a reciprocal influence of the molecules upon one another.

When, in the titration, we pass the point where the neutralization of the soap begins (about pH 10), the solution becomes colloidal. There appears a marked but not intense opalescence in the solution, which means that the fatty acid molecules rapidly aggregate to particles of colloidal size. This faint opalescence is not much altered within the pH range down to about 8.25. At this point, which is identical with the half neutralization point, a separation of oil droplets begins which makes the solution milky and soon quite opaque.

A fatty acid molecule has a length of about  $25 \cdot 10^{-8}$  cm. If the particles shall give opalescence, the size must be at least half the wave length of visible light, i. e. about  $4000 \cdot 10^{-8}$  cm. The largest diameter of the particle must therefore contain at least 160 molecules of fatty acid. If the particle is spherical in shape this corresponds to  $2.14 \cdot 10^6$  molecules in the particle.

The number of particles ( $n$ ) present in the surface of a spheric body is in relation to the total number of particles ( $N$ ) given by the formula:

$$n = N^{\frac{2}{3}}$$

In the colloidal particle of fatty acid in the example stated above we have the number of surface particles

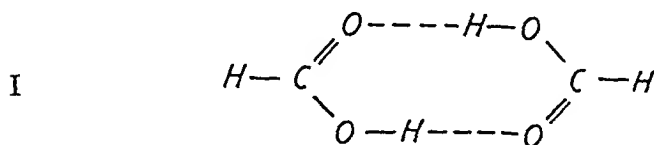
$$n = (\sqrt[3]{2.14 \cdot 10^6})^2 = 1.66 \cdot 10^4$$

i. e. less than one per cent of the molecules are placed in the surface of a spheric particle of the given size.

In the introductory chapter it was mentioned that *Frazer* found that lipid particles of a size of about  $0.5 \mu$  ( $= 5\,000 \cdot 10^{-8} \text{ cm}$ ) may pass the intestinal membrane when their surface is made hydrophilic. The bile acid molecules have a high polarity, and if they are placed at the surface of the fatty acid particles with their hydrophilic ends orientated against the water phase, a relatively small quantity is sufficient to make the particle hydrophilic. According to the above example the quantity of bile acid needed when the total surface of the particle has to be covered is of an order of magnitude of less than one per cent of the number of fatty acid molecules.

During the titration a relatively faint opalescence is retained by the solution until one half of the salt is neutralized. When the half neutralization point is passed, each further quantity of HCl added makes the solution more milky and opaque. The particles increase markedly in size and if the solution is left undisturbed larger oil droplets assemble on the surface. Before this point the solution has a transparency which is not much altered during the titration. That there is really a change in the condition at this point also appears from the slight, though marked, inflexion of the curve at this point.

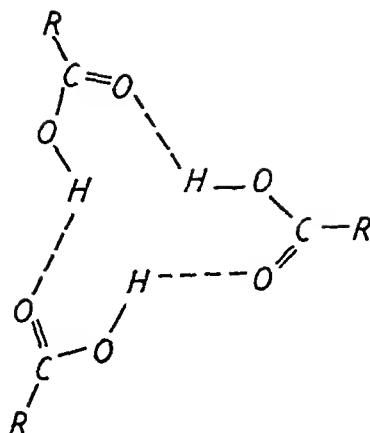
The lower fatty acids have a great tendency to occur in a dimerous form, in which two molecules combine by means of co-ordinate linkage or hydrogen bonds. As an example is given the formula for the associated formic acid quoted from *Bastiansen, Finbak and Hassel* (1944):



The constitution is verified by electron interference spectra.

(Perhaps also a trimerous form of the fatty acid can occur. For the following reasonings it makes no essential difference which of the forms is formed).

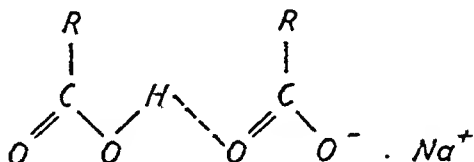
II



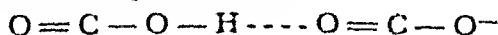
The long chain fatty acids have not been investigated in this respect. If the long chain acids aggregate in the same way, the dimerous (or trimerous) form must appear as electro-neutral particles and the long hydrocarbon chains (R) must make them extremely insoluble in water. Further the hydrocarbon chains must show great mutual attraction not unlike that of simple aliphatic hydrocarbons, and tend to aggregate to particles of large size.

It is quite clear that in a soap — fatty acid system the acid has not so great a tendency to form large drops (confer the above). It is well known that the fatty acids do not form basic salts (*McBain & Martin*, 1914), but they form well defined and crystallisable acid salts (acid soaps) (*McBain & Stewart*, 1927). At the half neutralization point of the soap almost the total amount of fatty acid may be present as acid soap, and when passing this point the separation of larger droplets of the acids begins. The constitution of the acid soap might be:

III



where the negative charges are balanced by the sodium ions of the system. (The  $\text{Na}^+$  cannot replace one of the protons of the hydrogen bonds in formula I, since the sodium has no co-valences). Between the two oxygen atoms of different charge ( $=\text{O}$  and  $-\text{O}^-$ ) there may be resonance through the chain:



In this way the dimerous particle may obtain a relatively high stability, and the relatively low tendency of the fatty acid to separate out in the beginning of the neutralization of the soap is explained.

The dissolving power of sulfonic acids and bile acids.

The bile acids have the property to keep fatty acids in watery solution.

It might perhaps be expected that when the fatty acids are prevented from separating out and are kept in solution, they might be dissociated to a higher degree and attain a higher acid activity. Since the bile acids are weak acids they interfere with the electrometric titration of other weak acids.

It is well known that the fatty acids are also soluble in solutions of sodium salts of fatty acid chain sulfonic acids. Since the sulfonic acids are very strong acids they (or their sodium salts) do not interfere with the electrometric titration of weak acids. (The influence of the change in ionic strength is quantitatively of no importance in these experiments).

A titration curve for oleic acid with the equivalent amount of sulfonic acid added had not the same form as that of the pure oleic acid. (The sulfonic acid used was the sodium salt of dodecanoic sulfonic acid,  $n\text{-C}_{12}\text{H}_{25}\text{SO}_3\text{Na}$ ). The effect of the addition is seen in fig. 3. In its total range the curve is displaced in relation to the curve obtained for pure oleic acid. From this we see that when the fatty acid does not separate out, the active concentration of the fatty acid is apparently altered.

For the present investigation the acid range of the curve is of most interest. In the acid part there is a markedly higher proportion of soap present when sulfonic acid has been added.

Since the bile acids have many properties in common with the sulfonic acids and are also known to keep fatty acids in solution, it is very probable that they have a similar effect upon the fatty acid—soap systems.

Unfortunately it is very difficult to carry out the corresponding experiment with bile acids. Because of the weak acid nature of the bile acids an electrometric titration of fatty acids in solutions of bile acids will give an addition of the titration curves, and it will be difficult to resolve the observed curve into the component curves.

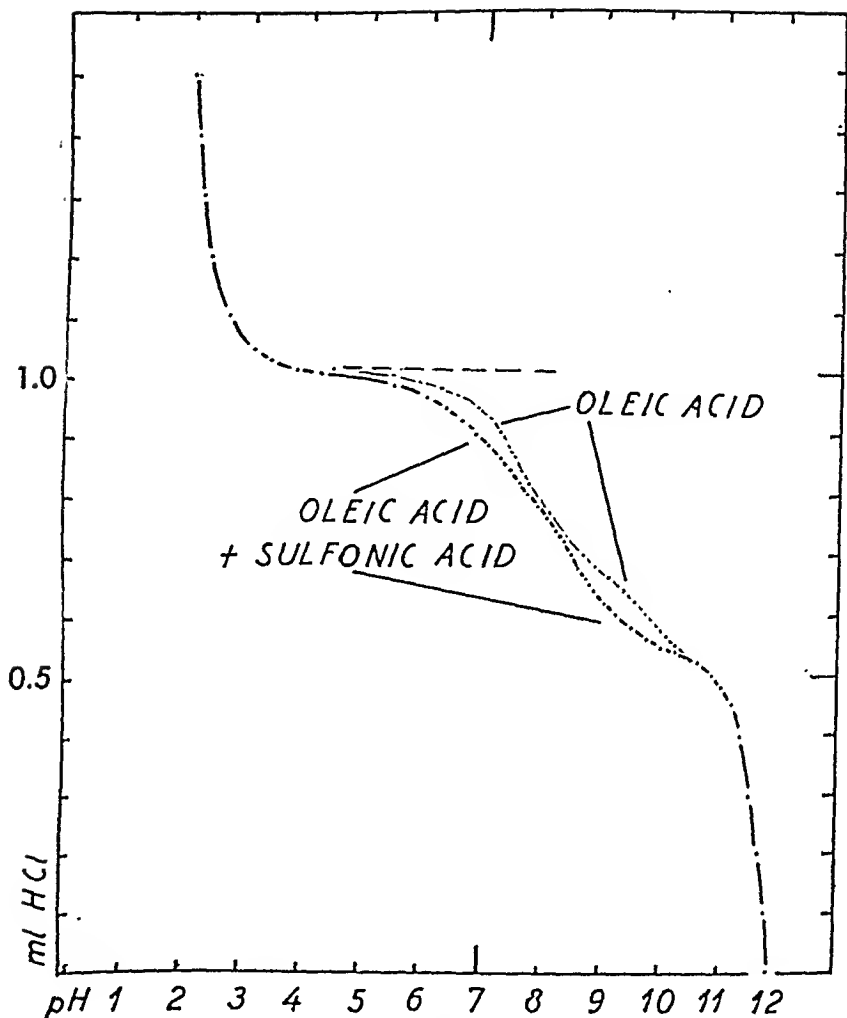


FIG. 3.

Electrometric titration of 0.5 mE of oleic acid + 0.5 mE of the sodium salt of dodecanoic sulfonic acid + 1 mE NaOH.

HOW LARGE A PROPORTION OF THE FATTY ACID IS PRESENT AS SOAPS AT THE REACTION OF THE INTESTINE?

The pure soap-fatty acid system.

In fig. 2 a curve is drawn which gives the theoretical titration curve for an acid with the  $K_a = 6.4 \cdot 10^{-9}$  ( $pK_a = 8.2$ ). It is seen that the inclination of the titration curve of the oleic acid is another, i.e. the  $K_a$  is not constant during the titration. In the

beginning the  $C_H+$  is smaller (pH higher) than that of the theoretical curve, which means that the  $K'_a$  is smaller than  $6.4 \cdot 10^{-9}$ . In the acid part of the curve the  $K'_a$  is larger than that of the theoretical curve. This fact makes it impossible from the  $pK'_a$  value of the half neutralization point to calculate anything concerning the quantities present as salt and acid respectively at the end range of the titration curve. Therefore we must try to judge the quantities present as acid and salt respectively from the graphs.

From the curve in fig. 2 it may directly be seen how large a proportion of the fatty acid is present as the sodium salt at a given pH. At  $pH=7$  it is found to be 0.07 mE of the total 0.5 mE, at  $pH=6$  it is 0.025 mE of the 0.5 mE, i. e. 14 per cent and 5 per cent respectively of the fatty acid present as soap.

#### Proportion as soap when sulfonic acid is added.

The similar calculation carried out for the system to which sulfonic acid was added gives the following result:

At  $pH=7$  0.12 mE out of the total 0.5 mE of fatty acid is present as soap, at  $pH=6$  0.05 mE is present as soap. This gives 25 per cent and 10 per cent respectively of the fatty acid present as soap.

When we find so large a proportion of the fatty acids present as soaps at the reaction of the intestine, it is not difficult to imagine that the absorption may take place in the form of soaps. A fair proportion of soap will always be present if the equilibria are restored at a fairly rapid rate.

If the fatty acids have separated out, the formation of soaps perhaps is a slow process according to the relatively low surface area of the particles. But in the normal intestine there are always considerable amounts of bile salts present, which keep a fairly high proportion of the fatty acids in solution. It is very probable that the bile acids in this way support the transmission of fatty acids to soaps in addition to the described ability of sulfonic acids to displace the proportion of soap-fatty acid in aqueous solution in favour of the soaps.



# THE FORMATION OF PHOSPHOLIPIDS IN THE INTESTINAL WALL

## IS THERE AN INCREASED AMOUNT OF PHOSPHOLIPIDS IN THE INTESTINE DURING FAT ABSORPTION?

### Analyses of the total small intestine.

It has repeatedly been demonstrated that during fat absorption there is an increased amount of phospholipids in the lymph flowing from the intestine, as well as in the blood.

In my own experiments I tried to find if fat absorption is followed by an increased amount of phospholipid in the intestinal wall. The results given in table 7 give no evidence for this assumption.

TABLE 7.

*Phospholipid and fatty acid content of small intestine in rat.*

	Weight gram	Duration of fat uptake, hours	$\mu$ Mol lipid-P per gram intestine	$\mu$ Mol fatty acid per gram intestine	Fraction of fatty acids as lecithin, per cent
Control animals	120		83	302	55
	295		53	270	39
	175		93	431	43
2.5 g peanut oil by stomach tube	140	2 $\frac{1}{2}$	82	599	27
	235	3	71	638	22
	240	6	66	887	15
	260	9	49	551	18
	245	9	41	464	18

Consequently, a chemical analysis of the total small intestine gives no information concerning the possible formation of phospholipids during the absorption of fat.

On the other hand there is found a considerable increase in the amount of fatty acids, which may be ascribed to a rise in either the neutral fat or fatty acid fraction of the lipids. This is most easily correlated to the well known histological picture of fat absorption in which the epithelial cells, the stroma of the villi, and the lymph vessels are filled with fat droplets.

### Analyses of small parts of the intestine.

If the fat absorption takes place by preference in a limited part of the small intestine, the possible changes in the lipids in this part

may be hidden by the large proportion of lipids in the other parts when the small intestine is analysed in toto.

Lipid substances are not equally taken up by all parts of the small intestine. It is well known that proximal to the openings of the bile duct and the pancreatic duct, the lymph vessels do not appear milky during fat absorption. Further *Croner* (1910) found differences in the absorption of soaps from different parts of the intestine (dogs), — in the upper parts soaps were not taken up but well in the lower parts. Fat was easily taken up in the lower parts, but also in the upper parts. *Mellanby* (1927, 1) found rapid absorption of fat emulsions in duodenum and slow absorption in ileum (cat).

In order to try to find a possible accumulation of phospholipids in a single part, analyses were carried out on relatively small parts of the intestine.

Two groups of rats were examined, the first one fasting control animals, the second one rats fed peanut oil by stomach tube. When fat uptake was at its maximum (about 6 hours after fat feeding) the small intestine was taken out, washed carefully and dried in vacuo. The gut was then divided into ten about equal parts, each part weighed and analysed separately.

In each sample the content of fatty acid and of lipid combined phosphorus was determined. The results are given in molar figures, which permits a direct comparison of the quantities of fatty acid and phosphorus. Presuming that all the lipid phosphorus is lecithin (i. e. each phosphorus atom combined with two fatty acid molecules) it is calculated how much of the fatty acid is combined with phosphorus. The surplus is probably mostly neutral fat, but is given as fatty acid in the tables.

In tables 8 and 9 the analytical results from seven normal rats are given. It will be seen that in the individual rat there are very considerable variations in the phospholipid as well as in the fatty acid content of different parts of the intestine. In the single rat the variations are not much smaller than between the different animals.

On the other hand, the average values for the seven rats show a marked regularity. The phospholipid content (table 8) seems to be a little lower in the distal part. The fatty acid content (table 9) seems to be a little lower in the proximal part, highest in the middle, and again somewhat lower in the distal part. For the fatty acids the irregularities in the single animals are not so pronounced, and the

TABLE 8.

*Phospholipid content of the small intestine of rats. Control animals.*Figures given in  $\mu$ Mol lipid phosphorus per g intestine, dry weight.

Rat No.	Parts of the intestine									
	1	2	3	4	5	6	7	8	9	10
1	76	85	95	91	84	97	72	69	76	43
2	52	47	61	85	58	80	34	34	33	43
3	95	100	101	98	97	98	82	77	58	92
4	48	46	37	36	63	71	31	31	34	30
5	33	43	42	35	59	65	33	26	28	23
6	53	72	47	50	39	36	33	32	28	39
7	42	39	46	41	27	30	21	23	27	29
Average,	57	62	61	62	61	68	44	42	41	43
$\mu$ Mol P/g intestine	$\pm 8$	$\pm 8$	$\pm 10$	$\pm 10$	$\pm 9$	$\pm 9$	$\pm 9$	$\pm 8$	$\pm 7$	$\pm 9$

tendency towards lower values in the end parts may be seen in most of them.

The fraction of fatty acids present as phospholipid (table 10) varies between  $\frac{1}{2}$  and  $\frac{1}{3}$  of the total. Perhaps the relative fraction of phospholipids is a little lower in the distal part, but on the whole we can say that no marked differences appear in the different parts of the intestine in the untreated animals.

TABLE 9.

*Fatty acid content of the small intestine of rats. Control animals.*Figures given in  $\mu$ Mol fatty acid per g intestine, dry weight.

Rat No.	Parts of the intestine									
	1	2	3	4	5	6	7	8	9	10
1	80	160	130	150	150	180	170	160	190	200
2	220	250	240	280	320	310	330	300	230	260
3	300	370	340	500	540	680	550	450	330	180
4	250	320	300	260	340	340	290	220	220	230
5	160	180	160	190	280	320	200	270	230	100
6	170	200	190	180	270	220	190	200	190	200
7	400	370	350	230	290	250	240	220	200	210
Average,	230	260	240	260	310	330	280	260	230	200
$\mu$ Mol fatty acid/g intestine	$\pm 40$	$\pm 30$	$\pm 30$	$\pm 40$	$\pm 40$	$\pm 60$	$\pm 30$	$\pm 40$	$\pm 20$	$\pm 20$

TABLE 10.

*Fraction of the fatty acids in rats intestine present as phospholipids.  
Control animals.*

Figures given in per cent of total fatty acids.

	Parts of the intestine									
	1	2	3	4	5	6	7	8	9	10
% Phospholipids .....	50	48	51	48	39	41	31	32	36	43

Tables 11, 12 and 13 show the corresponding figures obtained from rats intestine during fat absorption. The rats were fed 2.5 gram peanut oil by stomach tube 6 hours before the animals were killed. In all cases the fat uptake was normal with the mesenteric lymphatics milky white.

It will be seen that even in these rats there are considerable irregularities between the different parts of the intestine of the individual rat, and also between the different rats.

The average values show, when compared with the untreated animals, no increase in the phospholipid content after a fat meal. No single part of the small intestine shows a phospholipid content higher than normally.

The fatty acid content, on the other hand, is found to increase considerably during fat absorption, and this fact refers to all parts of the intestine. The distribution of the fatty acids in the single

TABLE 11.

*Phospholipid content of the small intestine of rats during fat absorption.*

Figures given in  $\mu$  Mol lipid phosphorus per g intestine, dry weight.

Rat No.	Parts of the intestine									
	1	2	3	4	5	6	7	8	9	10
1	61	80	86	84	80	88	80	78	90	74
2	73	58	80	79	61	53	68	69	54	37
3	75	63	74	86	60	57	57	57	64	64
4	59	53	51	52	53	48	50	43	54	41
5	65	42	48	45	41	28	30	25	41	42
6	52	58	66	58	50	45	38	29	36	25
7	40	60	60	52	54	51	46	46	41	47
Average, $\mu$ Mol P/g intestine	61 $\pm 5$	58 $\pm 4$	66 $\pm 5$	65 $\pm 6$	57 $\pm 5$	53 $\pm 7$	53 $\pm 7$	50 $\pm 7$	54 $\pm 7$	47 $\pm 6$

TABLE 12.

*Fatty acid content of the small intestine of rats during fat absorption.*Figures given in  $\mu$  Mol fatty acid per g intestine, dry weight.

Rat No.	Parts of the intestine									
	1	2	3	4	5	6	7	8	9	10
1	430	660	540	880	910	750	680	580	510	380
2	360	530	600	440	870	760	1 000	710	570	400
3	710	710	880	1 020	1 240	1 260	1 180	1 110	640	260
4	390	460	510	390	330	670	710	710	700	590
5	350	520	410	380	530	720	410	450	480	360
6	410	390	390	580	610	760	640	610	570	500
7	380	420	420	530	530	410	530	710	520	420
Average,	430	530	540	600	720	760	740	700	570	420
$\mu$ Mol fatty acid/g intestine	$\pm 50$	$\pm 50$	$\pm 60$	$\pm 100$	$\pm 110$	$\pm 100$	$\pm 70$	$\pm 80$	$\pm 30$	$\pm 40$

parts are like that in the untreated animals. There is a distinct increase from the proximal end toward the middle, and again a decrease toward the distal parts.

Since the fatty acid content is high, the fraction of it present as phospholipid (table 13) is lower than normally, about the half of the normal. The increase in the fatty acid content is therefore probably due to a higher content of neutral fat. As mentioned before, this fits in very well with the well known histological picture of fat absorption.

In table 14 are given the amounts of fatty acid present as non phospholipid substance (probably most of it as neutral fat). The fatty acid fraction present as phospholipids is calculated as if all of it were lecithin. It is seen that the increase during fat absorption is about 3 times that of normal resting intestine. Not only is the absolute increase larger in the middle parts, but also the relative increase is higher in the middle parts of the intestine.

TABLE 13.

*Fraction of the fatty acids in rats intestine present as phospholipids during fat absorption.*

Figures given in per cent of total fatty acids.

	1	2	3	4	5	6	7	8	9	10
% Phospholipids .....	28	22	24	22	16	14	14	14	19	22

TABLE 14.

*Increase in non-phospholipid fat of the intestine during fat absorption.*Figures given in  $\mu$ Mol fatty acid per g intestine.

Parts of the intestine	1	2	3	4	5	6	7	8	9	10
Non absorbing intestine ...	12	14	12	14	19	19	19	18	15	12
Fat    »                    » ...	31	41	41	47	61	65	63	60	46	33
Difference = increase during fat absorption .....	19	27	29	33	42	46	44	42	31	21
Increase factor .....	2.6	2.9	3.4	3.4	3.2	3.4	3.3	3.3	3.1	2.8

### THE FORMATION OF PHOSPHOLIPID FOLLOWED WITH RADIO-ACTIVE PHOSPHORUS AS INDICATOR

#### General and technique.

Even if there is no increase in phospholipid content in the intestinal wall, there may be a considerable synthesis of phospholipids during fat absorption. The phospholipid may be transported away or it may be broken down again. The formation of new phospholipid molecules may to some extent be followed by introducing labelled atoms of phosphorus (i. e. radioactive phosphorus,  $^{32}\text{P}$ ).

The metabolism of phosphorus has been extensively investigated by Hevesy and his many collaborators. Concerning the phospholipids of different organs he found the most rapid synthesis in the liver and the intestine. It is of interest, in regard of the theories of phospholipid formation during fat absorption, to investigate whether the rate of formation of phospholipids in the intestine is changed during fat uptake.

If, when  $^{32}\text{P}$  is administered to the experimental animal, we find a higher radioactivity in the phospholipids during fat uptake, it is evident that a larger amount of phospholipids is new synthesized.

Experiments on this matter were carried out on rats. The rate of formation of phospholipids found in different individuals is probably liable to variations. In view of the limited amounts of  $^{32}\text{P}$  available, it was impossible to carry out experiments to find normal values on a larger number of animals, and it was further impossible to carry out the absorption experiments on a number of animals sufficient to give reliable average values. Therefore, the possible

variations in different individuals were to some extent avoided by comparison of the function of different loops of the intestine of one and the same animal.

The results mentioned in the preceding chapter indicate that no considerable differences in the ability of phospholipid formation can be expected in any special part of the intestine, but nevertheless the most proximal and distal parts were not used.

Relatively small loops of the intestine were isolated by ligature, some of them being used for fat uptake, the other ones serving as non-absorbing controls from the same animal.

The experiments were carried out on young white rats of about 150 g weight.

The  $^{32}\text{P}$  was given as intramuscular injections of sodium phosphate, which is almost completely absorbed and evenly distributed in the blood within 15 minutes. The  $^{32}\text{P}$  was given at different times in relation to the end of the fat absorption experiment. When a specially rapid absorption and distribution of the  $^{32}\text{P}$  was desired, the injection was given intracardially.

All operations were carried out in urethane narcosis. When the abdomen is opened in the mid line, there is almost no bleeding, and precautions against blood loss are not necessary. With ligature silk loops of the small intestine, each of them about 2—3 cm in length, were carefully isolated. Much care ought to be taken to place the ligatures on such places that the blood circulation of the small loops remains as far as possible undisturbed. The operations were carried out in a room heated to 35° C and nearly saturated with moisture. To prevent injury of the intestine by evaporation, there was further throughout the experiment dripped physiological saline on it.

Alternate intestine loops were used for absorption of fatty acid, the intermediary loops used as simultaneous controls. Emulsions of oleic acid (1 per cent) in sodium taurocholate solution (4 per cent) were injected into the loops through a thin cannula, the corresponding taurocholate solution without oleic acid into the control loops.

When all the loops are not to be taken out simultaneously, the blood vessels leading to the loop to be cut out must be tied, else the animal bleeds to death.

The piece cut out is distended on a cork plate and frozen with  $\text{CO}_2$ -ice to stop possible metabolic processes in it. It is then rapidly dried in vacuo, afterwards the extraction of the lipids is made with an alcohol-ether mixture (equal parts of absolute alcohol and ethyl ether).

### Absorption of oleic acid.

In the graphs given in figures 4 to 8 the radioactivity is given in arbitrary units calculated per mg intestine. The quantity of  $^{32}\text{P}$  administered, the experimental time, and consequently the serum activity etc. have not been constant in the different experiments, and the absolute values for the radioactivity cannot therefore be compared in the different experiments.

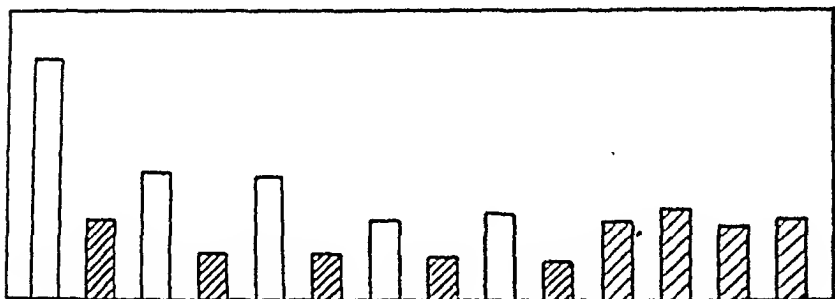


FIG. 4.

Radioactivity of phospholipids from rats intestine given in arbitrary units per gram dry intestine. Animal killed 14 minutes after intracardial injection of  $^{32}\text{P}$ .

White columns: loops absorbing oleic acid, hatched columns: control loops.

The four columns at the right end of the figure give the phospholipid activity found in intact intestine at the same time.

In the experiment shown in figure 4 the operation was carried out and the solutions injected into the loops  $\frac{1}{2}$  hour before the injection of  $^{32}\text{P}$ . In this way the intestine was fully engaged in fat uptake when the  $^{32}\text{P}$  reached it by the blood. The  $^{32}\text{P}$  was injected intracardially to secure a momentary distribution in the circulation. In order to control that the injection had really gone directly into the heart, the injection fluid was coloured by the harmless dyestuff T 1824. The animal was killed 14 minutes later.

It will be seen that in all the loops containing oleic acid, the lipid radioactivity is considerably in excess of that in the control loops.

The four columns at the right end of figure 4 show the lipid activity found in pieces from the unligatured parts of the intestine. The values are here rather constant and higher than in the ligatured control loops. Probably this is due to a better circulation in the intact intestine, but the result shows that the conditions in the isolated loops are not very far from normal.



In the experiment shown in figure 5 the operation was begun 2 hours after the injection of  $^{32}\text{P}$  and the experiment was finished 1 hour later.

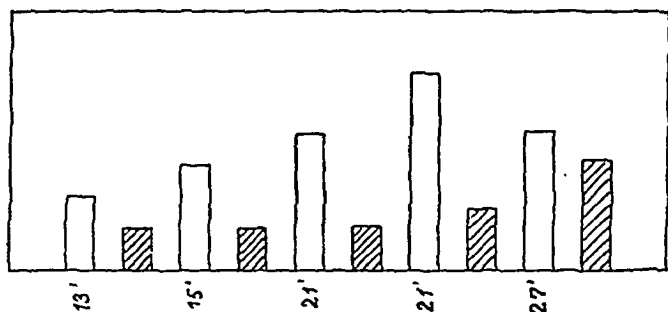


FIG. 5.

Radioactivity in phospholipids from rats intestine 3 hours after injection of  $^{32}\text{P}$ . Below the columns is given the time in minutes from the injection of oleic acid into the loops until the loops in question was cut ut.

White columns: loops absorbing oleic acid, hatched columns: control loops.

The loops were cut out at different times after the injection of the oleic acid emulsion. The loops having contained oleic acid showed a higher activity than the control loops, and the longer time the loops had contained the fatty acid, the higher activity was found.

The results of these two experiments are so uniform that a considerable larger synthesis of phospholipids seems to take place in the intestinal wall when it absorbs fatty acid.

The results of these experiments can be explained in two different ways:

- 1) An increase in the total amount of phospholipids in the intestine, or
- 2) An unaltered amount of phospholipids, but increase in its activity.

The analyses given on page 50 ff. give evidence against the first assumption, but since the blood circulation is probably not quite sufficient in these experiments, the transport of a supposed new-formed phospholipid away from the intestine could have been decreased.

The second assumption is the most probable. Phospholipids are synthesized, but they are removed at about the same rate as they are formed. They could be removed by splitting or they could be transported away from the intestine.

From *Hevesys* experiments it is known that the phospholipid normally present in the intestine has exchanged its phosphorus in about 24 hours. Therefore the phospholipid in the control loops in experiment 2 (two hours after  $^{32}\text{P}$  administration) have not reached the activity of the serum. Consequently, if phospholipid is new synthesized from its components it must have a considerably higher activity. This new formed phospholipid could very well be imagined, to some extent, to mix with and to "wash out" some of the less active phospholipid already present, whether it is brought away by transport from the intestine or by splitting.

If we accept this hypothesis, we still don't know if there is formed phospholipid which is transported away, or if the synthesis is only an intermediary process in the metabolism of the cells, e. g. a temporary process in the transport of the fatty acids through the border of the epithelial cells from the content of the intestinal lumen towards the basis of the cells.

A determination of the phosphorus content of the lipid samples permits a calculation of the activity of the phosphorus combined in the lipids (specific activity of the phosphorus).

Unfortunately the analyses failed in this special case and gave an uncertainty of about 20 per cent on the results. The specific activity, calculated as activity per mg lipid phosphorus, gave the following figures:

loops absorbing oleic acid .....	$0.96 \pm 0.12$
control loops .....	$0.65 \pm 0.18$

The figures show a somewhat lower specific activity in the control loops, but the difference is not statistically significant.

In figure 6 is given an experiment which is carried out like that in figure 5, but the  $^{32}\text{P}$  was injected eight days before the absorption experiment was carried out. We find almost the same activities in all the loops, either they have contained fatty acid or not.



FIG. 6.

Radioactivity in phospholipids from rats intestine 8 days after injection of  $^{32}\text{P}$ .  
White columns: loops absorbing oleic acid, hatched columns: control loops.

The result is easily explained. From *Hevesy's* work it is known that the phosphorus of the lipids in the intestine reaches equilibrium with the phosphorus of serum in about 24 hours. The phospholipids present in the intestine before the absorption experiment are consequently formed between the 7th and 8th day after the  $^{32}\text{P}$  was introduced. After so long time the level of  $^{32}\text{P}$  is not much altered within one day. The phosphorus of the new synthesized lipids has therefore almost the same specific activity as that of those already present. We know that no increase in the total amount of phospholipids can be expected, and consequently we find the same activity in fat absorbing and non absorbing loops.

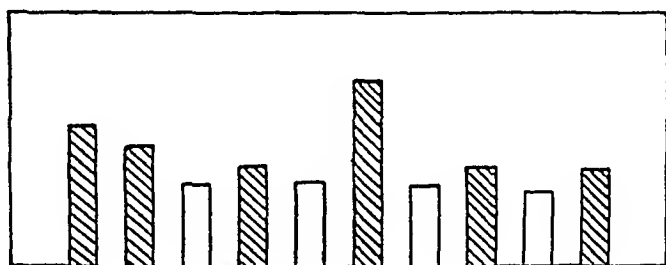


FIG. 7.

Radioactivity in phospholipids from rats intestine 28 hours after injection of  $^{32}\text{P}$ .  
White columns: loops absorbing oleic acid, hatched columns: control loops.

Figure 7 shows an experiment carried out 28 hours after the injection of  $^{32}\text{P}$ . The results are somewhat irregular, but on the whole the oleic acid containing loops have a lower and more constant activity level. The result can be explained by a reasoning similar to the above.

At this point of time the activity level of the serum is low as compared with that in the foregoing day and night, and the slope in the curve of serum activity is still steep. The phospholipids present in the intestine have therefore a higher activity than the new ones formed during the experiment with the relatively low activity level.

#### Phlorrhizin poisoning.

In the introductory chapter the phosphorylation theories of *Verzar* were mentioned (p. 22 ff.). His experimental attempts to influence phospholipid formation by poisoning with monoiodoacetic acid or phlorrhizin were discussed and to some extent criticized.

A single experiment was carried out to control *Verzar's* work. The

chief object in this experiment was to avoid the general poisoning of the animals which was produced in the experiments carried out by *Verzar* (subcutaneous injection of the drugs). This intention was realized by applying the poison directly in the intestine. In this way the intestine may be exposed to a relatively high concentration of the poison, and nevertheless a poor general condition of the animal is avoided.

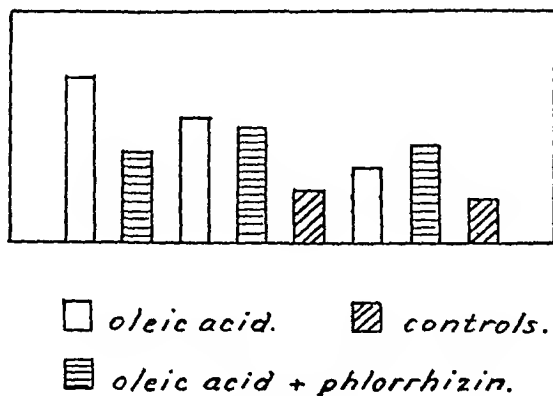


FIG. 8.

Radioactivity in phospholipids from rats intestine in which some of the loops were poisoned with phlorrhizin.

The experiment shown in figure 8 was carried out with the technique of a series of isolated loops of the intestine. The concentration of phlorrhizin applied was 0.02 molar (about 1 per cent) as used by *Carlsen* (1945) in work on amino acid and glucose absorption. In some of the isolated intestinal loops were injected an oleic acid emulsion, in others the same emulsion with the phlorrhizin added. The control loops contained pure sodium taurocholate solution.

On the narcotized rat, the loops were isolated and the solutions injected into them. It was assumed that after 15 minutes the absorption of the fatty acids would be running and that the poisoning would have developed in the loops containing phlorrhizin. At this point of time the  $^{32}\text{P}$  was injected intracardially, and 30 minutes later the animal was killed.

From the figure it is seen that the formation of phospholipids is of the same order of magnitude in the poisoned loops as in non poisoned, and in both of them considerably larger than in the controls. The formation of new phospholipids is, in this experiment, not prevented by phlorrhizin.

TABLE 15.

*Experiment showing the formation of phospholipids in phlorrhizin poisoned intestine.*

	Weight mg	Activity	Activity per gram intestine	$\mu$ Mol li- pid P per gram intestine	Specific activity
Loops containing oleic acid, in all .....	234	19.1	81	120	0.68
Loops containing oleic acid + phlorrhizin, in all .....	226	15.7	70	85	0.82
Control loops, in all .....	86	2.9	34	100	0.34

The numerical data of this experiment are summed up in table 15.

The lipid activity in the loops containing oleic acid is not significantly higher than in the poisoned ones. The specific activity of the lipid phosphorus is a little higher in the poisoned loops.

The experiment was repeated in just the same way. The results given in table 16 show results similar to the above. There is no definite difference in the formation of phospholipid in poisoned and non-poisoned loops. The control loops have formed much less of

TABLE 16.

*The formation of phospholipids in phlorrhizin poisoned intestine.*

	Lipid P activity per gram intestine		Specific activity of lipid P	
	Single loops	Mean	Single loops	Mean
Loops containing oleic acid	12.9		3.88	
	7.3	9.7	2.16	3.12
	8.8		5.31	
Loops containing oleic acid + phlorrhizin	9.8		2.65	
	10.8	9.1	2.45	2.96
	6.7		3.78	
Control loops	2.8		1.14	
	8.0	5.2	1.15	1.26
	4.7		1.49	

new phospholipids. Corresponding to this the specific activity is lower in the loops forming new phospholipids at the low rate.

From these experiments it will be seen that the rate of formation of phospholipids in the intestine is not depressed by phlorrhizin in a concentration which interferes heavily with glucose absorption.

#### Control experiments, absorption of glucose.

The main result of the experiments on absorption of oleic acid is that when the intestine absorbs oleic acid, phospholipids are formed at a considerably higher rate than in non absorbing intestine.

This increased rate of phospholipid formation may be due to a formation of phospholipids caused by the absorption of fatty acids, but another possibility cannot be excluded. When general absorption work is carried out, the metabolism of the cells increase and it might be suggested that this increase also interferes with the phospholipids. In other words, the turnover of phospholipids may follow an increase in the general metabolism of the intestine caused by absorption work.

If an increased activity gives increased phospholipid turnover, the absorption of glucose should give an effect similar to that of fatty acid absorption.

Experiments to investigate the effect of glucose absorption upon phospholipid formation, as compared with the effect of fatty acids, were carried out similar to the experiments described in the preceding chapter. In table 17 the results are given of an experiment in which solutions containing respectively oleic acid and glucose were injected into alternate loops of the small intestine.

The oleic acid emulsion was the same as that used in the experiments described above, 1 per cent oleic acid in 4 per cent Na-taurocholate solution. The glucose solution was a 0.15 molar (ca. 2.7 per cent) solution in 4 per cent Na-taurocholate (to make sure that no difference arises from a different bile acid content of the loops).

The figures in table 17 show that the total amount of phospholipids is the same in oleic acid absorbing and glucose absorbing loops. But the specific activity of the phospholipids is considerably higher in the loops absorbing oleic acid. The difference is about the same as that between oleic acid absorbing and non absorbing loops.

From this it will be seen that the phospholipid formation in glucose absorbing intestine is considerably lower than in fatty acid

TABLE 17.

*Formation of phospholipids in oleic acid absorbing intestine compared with glucose absorbing intestine.*

	$\mu$ Mol lipid P per gram intestine		Radioactivity of lipid P per gram intestine		Specific activity of lipid P	
	Single loops	Mean	Single loops	Mean	Single loops	Mean
Oleic acid absorption	48		108		2.24	
	47		141		3.01	
	65	$57.6 \pm 6.5$	125	$131.8 \pm 11.6$	1.91	$2.42 \pm 0.29$
	80		113		1.41	
	48		172		3.55	
Glucose absorption	32		63		1.96	
	48		76		1.57	
	45	$50.2 \pm 6.1$	103	$80.1 \pm 6.5$	2.30	$1.68 \pm 0.19$
	58		79		1.36	
	68		82		1.21	

absorbing, but the rate of phospholipid formation might still be somewhat increased compared with non absorbing intestine.

To see whether glucose absorption causes any rise in phospholipid turnover as compared with the resting intestine, an experiment was carried out where 0.3 molar glucose solution (ca 5.4 per cent) was injected into each second loop, the other loops left undisturbed. An other advantage of this course was that a possible harmful influence of the bile acid was avoided.

The result is given in table 18.

TABLE 18.

*Formation of phospholipids in glucose absorbing loops of the intestine compared with non absorbing loops.*

	$\mu$ Mol lipid P per gram intestine		Radioactivity of lipid P per gram intestine		Specific activity of lipid P	
	Single loops	Mean	Single loops	Mean	Single loops	Mean
Glucose absorbing loops	88		377		4.26	
	104	97	363	325	3.48	3.38
	98		236		2.41	
Non absorbing loops	99		342		3.47	
	94	109	314	317	3.34	3.13
	114		294		2.58	
Unligatured loops	119	108	319	356	3.67	3.85
	97		392		4.04	

No difference is found between the absorbing loops and the resting ones. For comparison two pieces of the non ligatured intestine was analysed. The activity in these pieces are a little higher, but the difference is not statistically significant. A certain difference would be expected, since the ligatures must unavoidably influence a little upon the blood circulation of the ligatured loops.

The increase in cell activity caused by glucose absorption brings about no rise in phospholipid turnover corresponding to the rise found in fatty acid absorption.

#### ATTEMPTS TO JUDGE THE RATE OF FORMATION OF PHOSPHOLIPIDS

The experiments on isolated loops of the intestine are not well suited for a calculation of the rate of phospholipid formation. The blood circulation can hardly be expected to be adequate in the loops, and further disturbances are brought about by the narcosis, the handling of the intestine, the displacement of it, the cooling etc, even if the drawbacks of these factors are kept as small as possible.

Therefore, a series of experiments were carried out on a number of intact animals.

Rats were given an ample amount of peanut oil (2.5 gram) by stomach tube. The fat absorption ought to be at its maximum after about 5 hours. At this time, when the hypothetical phospholipid synthesis runs at full speed, the  $^{32}\text{P}$  was injected intramuscularly.

In the series of experiments given in table 19 the animals were killed 15 minutes after the injection of  $^{32}\text{P}$ .

TABLE 19.  
*Radioactivity of phospholipids in fat absorbing and resting intestines respectively, 15 minutes after the administration of  $^{32}\text{P}$ .*

	Weight gram	Activity of the P-lipids per gram intestine	$\mu$ Mol lipid P per gram intestine	$\mu$ Mol fatty acid per gram intestine	Spec. act. of lipid P	Spec. act. of serum P	
Control	190	8.1	8.7	33	184	0.24	683
animals	180	9.3		43	201	0.22	750
2.5 g peanut	205	30.0	26.9	45	543	0.67	656
oil by	210	23.8		50	498	0.47	606
stomach tube							



The phospholipid found when the intestine is engaged in fat uptake has an activity about 3 times that found in the resting intestine. This means that during the 15 minutes the serum has contained  $^{32}\text{P}$ , a more extensive synthesis of phospholipid has taken place in the fat absorbing intestine.

The total amounts of phospholipid in the intestine are not very different from the normal values given in table 7 (page 50). The amount of fatty acid is also of the same magnitude as in table 7, i. e. a considerable increase in intestines absorbing fat.

During the 15 minutes the activity of the serum has been varying greatly. Generally, when injecting  $^{32}\text{P}$  we find a sudden increase for some minutes, a maximum, and then again a rapid decrease. Initially the fall is very rapid, later it becomes more and more slow. When the first 15 minutes have passed, the serum activity curve has reached the rapid decrease. Because we have no constant activity in the serum phosphorus, it is very difficult to calculate the quantities of phospholipids synthesized in the period.

If we compare the specific activity (activity per P atom given in arbitrary units) of the inorganic phosphorus of the serum and that of the lipid phosphorus in the intestine, we can form a conception of how much phospholipid can be synthesized using serum phosphorus as building material.

We find that the specific activity in the phospholipid from fat absorbing intestine is one thousandth part of that in serum. The specific activity of the phospholipids in the intestines of the control animals is about 1/3000 part of the specific activity of serum.

From this it is evident that only a minute fraction of the phospholipids can have been synthesized from its components using serum phosphorus as building material.

A series of experiments were carried out like those mentioned, but the animals lived 60 minutes after the injection of  $^{32}\text{P}$  (table 20).

When the animals were killed 60 minutes after the injection of  $^{32}\text{P}$ , there had been built up considerably more active phospholipid, but the proportion of phospholipid formed in fat absorbing intestine in relation to the resting intestine was of the same order of magnitude as in 15 minutes experiments. The relation between the quantities of phospholipid metabolized in fat absorbing and in non absorbing intestine is consequently about the same after 15 minutes and after 60 minutes.

When we compare the specific activity of the intestinal phospho-

TABLE 20.

*Radioactivity of phospholipids in fat absorbing and resting intestines respectively, 60 minutes after the administration of  $^{32}\text{P}$ .*

	Weight gram	Activity of the P-lipids per gram intestine	$\mu$ Mol lipid P per gram intestine	$\mu$ Mol fatty acid per gram intestine	Spec. act. of lipid P	Spec. act. of serum P
Control animals	160	76	37	262	2.1	2.35 } 311
	250	103	40	—	2.6	
2.5 g peanut oil by stomach tube	260	430	34	—	10.7	9.5 } 328
	225	413	50	—	8.3	

lipids with that of the serum, we find it to be 1/30 of the serum spec. activity. Since the spec. activity of the serum is decreasing at this moment, an unknown proportion of the phospholipid has been formed during the period of higher activity, and if the phospholipids were synthesized using serum phosphorus as building material, we can say nothing about the percentage of the phospholipids in the intestine renewed in the period.

Generally, if turnover rates are to be calculated, the activity level of the serum ought to be kept constant by continuous injection of the labelled phosphate. These relations are discussed in detail by *Hevesy and Hahn* (1940, 1). Because of several difficulties, some of them to be discussed in the following chapter, we may suppose that even if the serum activity is kept at a constant level during the experiments, such experiments will not give much further elucidation of the processes treated in this paper.

#### DISCUSSION OF $^{32}\text{P}$ EXPERIMENTS

If a phospholipid formation is necessary for fat absorption we have two main possibilities, viz.

1) Phospholipids are synthesized in large amounts from the absorbed fatty acids and transported away. This alternative requires a large amount of phosphorus, which ultimately must be taken from the serum phosphorus.

2) Phospholipids are synthesized in the epithelial cells of the intestine as an intermediate step in the uptake of fatty acids and resynthesis of neutral fat. The fatty acid — glycerol part of the phospholipid molecule may be split off, and the phosphorus containing part of the molecule still be left more or less undisturbed.

1) It is not probable that phospholipid is transported away at a very rapid rate. In this case very active phospholipid would be found in the lymph or blood leaving the intestine. The increase in blood phospholipid activity, found by *Hevesy & Lundsgaard* (1937) to be only moderate during fat absorption, speaks against this. In the writers experiments the activities of the blood lipids were always too small for counting them.

2) An approximate calculation gives some information concerning the sources of phosphorus if the phospholipid synthesis takes place using fresh phosphorus atoms.

If a rat of average size (200 gram) can digest and absorb, say, 1 gram fat in 4 hours, the absorption per hour is 250 mg, or approximately 1 mMol fatty acid per hour. (This value corresponds to the values given for dogs by *Arnschink* (1890).) The order of magnitude of the lipid phosphorus content in the total small intestine is less than 0.05 mMol, corresponding to less than 0.1 mMol fatty acid in the phospholipid (2 Moles fatty acid per Mole P). Consequently, the amount of phospholipid synthesized during fat absorption in 1 hour should be more than 10 times as large as the preexisting phospholipid.

The inorganic phosphorus content of the total small intestine is about  $25 \mu\text{Mol}$ , and cannot yield enough phosphorus for the synthesis unless every P atom can be used several times.

We know very little about the sources of phosphorus from which the phospholipid synthesis takes place.

We may suppose that the new formed phospholipids in the intestine are really synthesized by the tissue and not transported to it by the circulation. *Hevesy & Hahn* (1940, 2) introduced labelled phosphatides into the circulation of rabbits, and investigated the rate of penetration of the labelled molecules into different organs. They found, in experiments of 4 hours duration, that the penetration into the small intestine was very slow (1940, 2, p. 15). Therefore we may suppose that the relatively rapid increase in phospholipid activity in experiments of up to 1 hour in duration are due to processes going on in the intestinal tissue.

It is very improbable that the phospholipids are synthesized directly from the serum phosphorus. In all likelihood the phosphorus passes the cell wall before taking part in the synthesis, and the phosphorus used for synthesis cannot have an activity higher than that of the inorganic phosphorus in the cell.

TABLE 21.

*Relation between the specific activity of the serum P, cell inorganic P, and lipid P from the intestines from 3 rats. Arbitrary units.*

Serum P Spec. act.	Cell inorganic P Spec. act.		Lipid P Spec. act.	
	fat absorbing intestine	non absorbing intestine	fat absorbing intestine	non absorbing intestine
1000	131	136	7.8	3.3
1000	293	222	6.9	4.1
1000	—	263	—	2.9

In table 21 are given the specific activities of serum phosphorus, inorganic cell phosphorus and phospholipid phosphorus in the small intestines of three rats. We find that the phospholipid specific activity is about 5, i. e.  $1/200$  of the serum specific activity but as compared with the cell inorganic phosphorus its specific activity is  $1/40$ .

This value would be reached if the phospholipids were totally renewed in  $1/40$  part of the cells and unchanged in the others. This cannot be the real situation, because even in the resting intestine the turnover is relatively rapid. On the other hand, the epithelial cells make up such a small fraction of the total intestinal tissue that the phospholipid specific activity in them may very well be of the same magnitude as the inorganic phosphorus.

There is good reason to believe that the increase in phospholipid formation described in the preceding chapters is really essential in fatty acid absorption. Further we know that the main chemical processes connected with absorption are probably limited to the epithelial cells. If the increase found is caused by an increase only or mainly in the epithelial cells, the phospholipid turnover in these cells may be extremely rapid.

Further information on this point might be expected to be found by analyzing the mucosa or, if possible, the isolated epithelium.

## GENERAL DISCUSSION

The present investigation was carried out as an attempt to throw some light upon the chemical processes involved in fat absorption.

The most recent work on fat absorption (*Frazer*) shows that the absorption of fat may take place in the unsplit condition as minute droplets, but there is no doubt that the absorption of fat can also take place in the way that the fats are hydrolysed to fatty acids and glycerol and the components absorbed separately.

The fats are split by the lipolytic enzymes of the digestive juices. *Pflügers* theory was that the fatty acids then combine with the sodium bicarbonate of the pancreatic juice to form soaps. The fatty acids are in this way made water soluble and may be absorbed in this state. Later on it was stated that the reaction of the intestine was actually slightly acid, and *Pflügers* theory of soap formation was rejected. It was well known that soaps are split up when an acid is added, and it was taken for granted that no soap (or only infinitesimal quantities of soap) can exist at the pH of the intestine.

In the writers experiments it was shown that if an acid is added to a soap solution until the pH of the intestine is reached, there will still be a considerable fraction of the fatty acids present as soap. Therefore, the pH of the intestinal contents is not a hindrance to the absorption of fatty acids as soaps.

When fatty acids or soaps are introduced into the small intestine they are readily absorbed, esp. when bile acids are present. The bile acids have the property of keeping the fatty acids in aqueous solution. Therefore it has been supposed that the fatty acids are absorbed as a water soluble bile acid complex. This complex must be of considerable size because a number of bile acid molecules (3—8) is necessary for keeping one fatty acid molecule in

solution. The molecular weight of glycocholic or taurocholic acid is about 500, and the complex will therefore have a molecular weight of about 2000—4000. It is not easy to imagine that a complex of that size is well suited for crossing the epithelial border.

Even if the bile acid complex is not absorbed, it has a very great importance because the fatty acids are kept in solution. If soaps are absorbed from the intestinal content, the complex can immediately give off fatty acids and restore the soap-fatty acid equilibrium. This would not be the case if the fatty acids had separated out from the aqueous phase. It is in agreement with this conception that in absence of the bile the fat absorption is retarded.

Concerning the fate of the fatty acids which have entered the epithelial cells of the villi, we do not know much.

It has been suggested that the fatty acids are synthesized to phospholipids as a step in the further transport away from the epithelial cell. A considerable turnover in the phospholipids of the intestine during fat absorption was demonstrated by *Sinclair*. He found that if characteristic fatty acids are fed, a very considerable proportion of the fatty acids in the phospholipids of the intestine are found to be replaced by new fatty acid molecules. This was explained by the supposition that an extensively increased phospholipid synthesis was involved in the transport mechanism. It is not possible from these experiments to conclude that a phospholipid synthesis is a necessary process in fat transport. If the fatty acids of the phospholipids can dissociate off at all, the presence of other fatty acids in the neighbourhood will result in an exchange between the fatty acid molecules. It is not improbable that the phospholipids are dissociated to a minute extent, since it must be supposed that esterases of a suitable character probably are present in all cells. If the characteristic fatty acids are present in a cell at all, they will therefore also appear in the phospholipids.

The result of the present investigation, that the turnover of the phosphoric acid part of the phospholipid molecules in the intestine is also much increased during absorption of fatty acids, makes it probable that the phospholipid turnover is really a process of much importance to fatty acid uptake, perhaps essential in it.

It would be of interest to know in which part of the intestinal wall the most intense turnover takes place, and to know if only one single fraction of the phospholipids has the rapid turnover rate, or if all the different types of phospholipids are engaged.

An investigation of these problems will probably make possible a better founded conception of the mechanisms of fatty acid absorption.

At the present we can say that the fatty acids which are split off in the intestine by the action of lipase are kept in solution by the bile acids. A considerable fraction of the fatty acids in the intestinal content are present as soaps. It is more probable that the passage through the cell surface takes place in a molecularly dispersed form than as a bile acid complex of large size. It has been made very probable that a phosphorylation process is essential in the further transport of the fatty acids, possibly in resynthesizing them to neutral fat.

## S U M M A R Y

I. In the introductory chapter is given a review of the present state of conception of fat absorption in the mammalian intestine.

In the first years of this century the viewpoint was brought forward and made probable that fat absorption only takes place after total hydrolysis of the fat. The most important work supporting this theory and some facts speaking against it are mentioned.

The theory of total hydrolysis is not in agreement with the recent work of *Frazer*, whose most important publications are referred to in detail. *Frazer* found that paraffin oil, which cannot be hydrolysed or split up by the digestive process, may be absorbed when it is finely emulsified. Further his results led to the view that unhydrolysed fats are absorbed into the lymphatics but free fatty acids are absorbed into the blood vessels. *Frazer* showed that fatty acids are not absorbed as droplets, and his results are in agreement with the conception that chemical processes are essential in the fatty acid absorption.

II. In the technical part are described the analytical methods used in the present investigation.

The extraction of the lipids from the tissue samples is not carried out in the usual manner which requires a total removal of the lipids. Instead of this complicated process, the tissue sample is enclosed in an ampoule together with a known quantity of the extraction fluid. Aliquot parts of this are pipetted off for analysis, and afterwards the total amounts of extractible matter are calculated.

The adequacy of the method is tested on tissue samples containing phospholipids tagged with radioactive phosphorus.



The fatty acid titration is carried out according to the method previously described by the writer. Some simplifications in the procedure are described.

The quantitative determination of phospholipids is carried out by a phosphate analysis.

The technique for the measurement of radioactive substances as applied in biological research is described. The special technique developed for measurement of phospholipids without isolation of the phosphorus is described and the reliability of the method is demonstrated.

III. In the experimental part two main problems are treated, viz. the possible existence of a certain amount of soaps in the intestinal content, and the investigation of the hypothetical phosphorylation of the fatty acids during absorption.

The common conception is that soaps cannot exist at the pH of the intestine. *Pflügers* old theory that the fatty acids are absorbed as soaps has therefore been abandoned. From a chemical viewpoint this is not correct, there must always be a certain amount of the fatty acids present as soaps in the intestinal content.

The quantity present as soap cannot be calculated in the usual way according to the law of mass action, because the dissociation constants of higher fatty acids are not known. Therefore electro-metric titration curves were made for oleic acid as an example of a long chain fatty acid. It was shown that the fatty acid — soap system cannot be treated in the usual manner in calculations of the dissociation constants.

The titration curve takes another course if a paraffin chain sulfonic acid is added to the system to prevent the fatty acid from separating out during the titration.

From the curves the fraction of the fatty acid which is present as soap at a given pH, in casu the pH of the intestine, may be approximately calculated.

In the pure oleic acid system ca 14 per cent of the acid is present as soap at  $\text{pH} = 7$  and 5 per cent as soap at  $\text{pH} = 6$ . In the system to which sulfonic acid is added the corresponding fractions are ca 25 per cent and 10 per cent respectively.

It may be supposed that the bile acids have an effect similar

to that of the paraffin chain sulfonic acids, i. e. that when these acids are present in the intestinal content a considerable proportion of fatty acids are present as soaps.

The formation of phospholipids during absorption of fat was investigated by means of radioactive phosphorus ( $^{32}\text{P}$ ) as indicator.

It was not possible to find an increase in the total amount of phospholipid in any part of the intestine during fat absorption. Therefore, if a phospholipid synthesis takes place, the newformed phospholipids must either be transported away or split up again near the place of formation.

By the use of  $^{32}\text{P}$  it was shown that the formation of phospholipids is considerably higher when oleic acid is absorbed by the intestine than in the non-absorbing intestine.

Poisoning with phlorrhizin did not decrease the rate of formation of phospholipids.

The increase in phospholipid formation could not be ascribed to the general increase in cell activity, because intestinal loops absorbing glucose did not synthesize phospholipids at the rapid rate but at the same low rate as non-absorbing intestine.

The activity of the phosphorus built into the phospholipids (expressed as specific activity) gives some information concerning the rate of formation of the phospholipids. The turnover of phospholipids in resting intestine and in fat absorbing intestine takes place at different rates. The possibility of judging these rates from the present experimental material is discussed.

In the last chapter the general points in fat absorption are discussed in relation to the writers experiments.

## SUMMARY IN DANISH

### DANSK RESUMÉ

I indledningskapitlet gives der en oversigt over den nuværende opfattelse af fedtoptagelsen i pattedyrenes tarm.

I de første aar af dette aarhundrede blev det synspunkt fremsat og sandsynliggjort, at fedtoptagelse kun sker efter fuldstændig hydrolyse af fedtet. De vigtigste arbejder som støtter denne teori omtales, ligesom visse forhold der taler imod.

Teorien om fuldstændig hydrolyse kan ikke bringes i overensstemmelse med *Frazers* arbejde. *Frazers* vigtigste publikationer omtales detailleret. Han fandt at paraffinolie, som ikke kan hydrolyseres eller spaltes af fordøjelsen, kan optages naar den er fint emulgeret. Videre førte hans arbejde til det synspunkt at ikke hydrolyseret fedt transporteres fra tarmen gennem lymfekarrene medens fri fedtsyrer transporteres af blodkarrene. *Frazer* viste at fedtsyrer ikke optages som en emulsion, og hans resultater er i overensstemmelse med den opfattelse at kemiske processer er af væsentlig betydning ved fedtsyreoptagelsen.

Det arbejde som er udført for at undersøge de kemiske processer ved fedtoptagelsen diskuteres med særlig henblik paa teorien om at en fosforylering af fedtsyrerne er nødvendig for deres optagelse.

I den tekniske del beskrives analysemetoder.

Ekstraktionen af fedtstofferne fra vævsprøverne er ikke udført paa den sædvanlige maade, der kræver en fuldstændig fjernelse af lipoiderne. I stedet for denne komplicerede fremgangsmaade bliver vævsprøven lukket inde i en ampul sammen med en kendt mængde af opløsningsmidlet. Naar alikvote dele udtages til analyse, kan de totale mængder af lipoider derefter beregnes.

Metodens paalidelighed er prøvet paa vævsprøver som indeholder phospholipoider mærket med radioaktivt fosfor.

Fedtsyrebestemmelser blev udført efter en metode som tidligere er beskrevet af forfatteren. Nogle forenklinger af metoden er beskrevet.

Den kvantitative bestemmelse af fosfatider blev udført ved en fosfatanalyse.

Den teknik som bruges til maaling af radioaktive substanser i biologisk forskning beskrives. Den teknik som blev udviklet til maaling af phospholipoider uden isolering af fosforet beskrives, og metodens paalidelighed paavises.

I den eksperimentelle del behandles to hovedproblemer, nemlig muligheden af at der kan findes en vis mængde sæber i tarmindeholdet, og undersøgelsen af den hypotetiske fosforylering af fedtsyrer under optagelsen.

Den almindelige opfattelse er at der ikke kan findes sæber ved tarmindeholdets pH. *Pflügers* gamle teori om at fedtsyrer optages som sæber blev derfor opgivet. Fra et kemisk synspunkt er dette ikke korrekt, en given del af fedtsyrerne maa altid være tilstede som sæber i tarmindeholdet.

Den mængde der er tilstede som sæber kan ikke beregnes paa den sædvanlige maade ved brug af massevirkningsloven, fordi de højere fedtsyrers dissociationskonstanter ikke kendes. Derfor udførtes elektrometriske titreringer af oliesyre som eksempel paa en langkædet fedtsyre. Det vistes at systemet fedtsyre—sæbe ikke kan behandles paa den sædvanlige maade ved beregning af dissociationskonstanter.

Titreringskurven faar et andet forløb hvis der tilsættes en langkædet sulfonsyre til systemet som forhindrer fedtsyren i at udskilles under titreringen.

Ved hjælp af kurverne kan man tilnærmet beregne den mængde af fedtsyrerne som findes som sæber ved en given pH, i dette tilfælde tarmindeholdets pH.

I det rene oliesyresystem er 14 % af syren tilstede som sæbe ved  $\text{pH} = 7$  og 5 % som sæbe ved  $\text{pH} = 6$ . I det system som indeholder sulfonsyre er de tilsvarende mængder henholdsvis ca 25 % og 10 %.

Der er grund til at formode at galdesyrene har en lignende virkning som sulfonsyrerne, d. v. s. at naar galdesyren er tilstede i tarmindeholdet er en større mængde af fedtsyrerne tilstede som sæber.

Dannelsen af fosfatider under fedtoptagelsen er undersøgt med radioaktivt fosfor ( $^{32}\text{P}$ ) som indikator.

Det var ikke muligt at finde en forøget fosfatidmængde i nogen del af tarmen under fedtoptagelse. Hvis der sker en fosfatiddannelse maa de nydannede fosfatider enten transporteres fra tarmen eller spaltes igen i nærheden af det sted de er dannede.

Ved brug af  $^{32}\text{P}$  vistes det at der er en betydelig større dannelse af fosfatider i tarm som optager oliesyre end i tarm som ikke optager oliesyre.

Forgiftning med phlorrhizin nedsatte ikke dannelsen af fosfatider.

Forøgelsen i fosfatiddannelsen kunde ikke tilskrives en almindelig forøget celleaktivitet, fordi tarmslynger som optager glukose ikke danner fosfatider med den hastighed som fedtsyreoptagende tarm, men med den lave hastighed som ikke optagende tarm.

Aktiviteten i det fosfor som er indbygget i fosfatiderne (udtrykt som specifik aktivitet) giver nogen oplysning om den hastighed som fosfatiderne dannes med. Omsætningen af fosfatider i hvilende tarm og i fedtoptagende tarm foregaar med forskellig hastighed. Mulighederne for at bedømme disse hastigheder fra det foreliggende eksperimentelle materiale diskuteres.

I det sidste kapitel diskuteres hovedpunkterne i fedtoptagelsen i forbindelse med forfatterens experimentelle resultater.

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INTERACTION OF CENTROGENIC AND  
CHEMOREFLEX CONTROL OF BREATHING  
DURING OXYGEN DEFICIENCY  
AT REST

*By*

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MED. LIC.

S T O C K H O L M

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## I. Preface.

A number of earlier papers from this laboratory refer to the reflexogenic control of breathing. The present work, partly being based on these investigations, was carried out during the years 1944—1946.

It is a pleasure to record my sincere gratitude to the Director of this Laboratory, Professor U. S. v. EULER, for stimulating advice and constant encouragement throughout the experimental work.

I wish to express my great appreciation to C. M. Hesser, M. L., and A. Åström, M. K., for their reliable and interested assistance during the experiments.

Finally, my thanks are due to the technician, Mr E. A. Kejbo, for the manufacturing of several pieces of equipment and to Mr C.-D. Lundquist for some of the drawings in the text.

Economic support for the construction of the recording system and other experimental accessories was received by personal grants from the Swedish Medical Research Council, the foundation "Therese och Johan Anderssons Minne" and the Nobel foundation.

Stockholm, April 1946.

A. G. HILDING BJURSTEDT.

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## II. Introduction.

The theme of the present work is a study of some of the physico-chemical and physiological factors, by which the hypoxic hyperventilation is automatically initiated and maintained. The first part of the work constitutes a review of those investigations concerning hemo-respiratory functions in oxygen deficiency, from which the present work was derived. The purpose of the review is to give a running account of earlier experimental results and at the same time to give prominence to certain important correlations, which can be made between these results.

In the second part of the work the problem is defined. The central point of the problem, the interaction of centrogenic and reflexogenic chemical control of breathing during oxygen deficiency, is only very incompletely known. The fundamental importance of the chemoreflexes is firmly established, but on the rôle of the chemosensitive cells of the centers themselves there is continuous discussion. Information is lacking on the interaction of centrogenic and chemoreflex control of breathing during the course of prolonged exposure to constant ambient oxygen deficiency. Whether the chemoreflexes are of the same importance in the initial stage of hypoxic hyperventilation as in the later stages has not previously been discussed.

The third part of the work is an account of the author's investigations. The experimental procedure employed will be shown to have given the possibility of revealing fundamental principles concerning the mechanism of hypoxic hyperventilation, principles that would be missed by more direct approaches to the isolated centers or chemoreceptors, working by themselves. Essentially it consists in observing those respiratory reactions, which occur

as the result of a temporary blocking of peripheral chemosensory impulses after varying exposure to ambient oxygen deficiency, and in judging these respiratory reactions by the aid of simultaneous, direct and continuous recordings of the oxygen saturation and the pH of the circulating arterial blood.

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### **III. Review of Basic Investigations, from which the Present Work has been derived.**

In bringing together those results of previous works on hemo-respiratory functions in oxygen deficiency, which bear a fundamental relationship to the present work, it seems appropriate to the requirements of logic and lucidity to seek evidence upon three main points. They are dealt with in separate sections. When taken together, they readily resolve themselves into the problem of the present work, which will be introduced in the subsequent chapter. The review is not meant to be a mere digest of earlier papers, but rather an attempt to correlate the present knowledge concerning the importance of the chemoreflexes for the hypoxic hyperventilation with earlier theories on the chemical control of respiration in oxygen deficiency. Such correlations are not possible without a knowledge of the characteristic changes in the acid-base composition of the blood under the influence of oxygen deficiency. This subject will therefore be considered first.

A complete reference of the overwhelming material so far existent should not be demanded. The author has thought it unwise to risk confusion by painstakingly accounting for such papers, that will not contribute essentially to elucidating the earlier and present standpoints within the scope of this work. There are also large numbers of important observations on hypoxic reactions in breathing, made during the last war in conjunction with the rapidly growing medical research activities, related to aviation, the results of which are not yet available.

#### **1. Changes in the Acid-Base Balance of the Blood at Rest during prolonged Exposure to a tolerated, constant Degree of Oxygen Deficiency in the inspired Air.**

The now generally accepted conception of the acid-base balance of the blood is comparatively young. It originally developed as the result of the introduction into physiology of the dissociation

theory of *ARRHENIUS*. An account of its evolution is not indicated here, but a few points of the greatest importance for the interpretation of changes in the acid-base composition of the blood in oxygen deficiency will be considered in the following historical abstract.

Before the main subject of the present section is dealt with, a short account will be given of the classification employed.

### Historical.

The outstanding features of acidosis are the decrease of the plasma bicarbonates and the increase of breathing. These symptoms were noticed by *WALTER* (1877) in his classic observations on animals, which had been given hydrochloric acid by stomach. *ARAKI* (1894) found considerable amounts of lactic acid in the urine from animals that had been partially asphyxiated with carbon monoxide. Together these observations constituted the basis for the earlier universal belief, that in oxygen deficiency, whatever its degree and duration, the oxidation of glucose should be hampered and lactic acid thereby liberated in the blood.

In 1904 *GALEOTTI* found that at high altitudes the "titration alkalinity" of the blood is markedly decreased. This finding seemed to confirm the general conviction, that oxygen deficiency causes a decrease of the alkalinity of the blood. For conditions of decreased "titration alkalinity" of the blood were at the time simply interpreted as decreased alkalinity, although often there must have been decreased hydrogen ion concentration, *i. e.* increased alkalinity. The important fact that the degree of alkalinity in the blood is not only determined by its buffering capacity against acids, but also by the actually prevailing carbon dioxide pressure was not understood.

This simple conception of decreased alkalinity in oxygen deficiency was later employed by *BOYCOTT & HALDANE* (1908), who advanced the theory that the increased respiratory activity under the influence of oxygen deficiency should be the result of lactic acid acidosis, lactic acid exerting its action in much the same way as carbon dioxide. As a secondary result an excessive blowing off of carbon dioxide should occur. Later on *WINTERSTEIN*'s first "reaction theory" (1911) actually demanded an implication of acidosis for an explanation of the hypoxic hyperventilation. The activity of the respiratory center was supposed to be controlled through an assumed decrease in the alkalinity of the blood.

Using the modern classification the main change in the acid-base composition of the blood was at the time believed to be a metabolic acidosis (*cf.* below).

In 1909 *L. J. HENDERSON* introduced the dissociation theory of *ARRHENIUS* into physiology. He applied the modern chemical conception of ionic equilibrium and hydrogen ion concentration to the blood. He showed that in the acid-base balance of the blood the acid is chiefly

carbonic acid ( $\text{H}_2\text{CO}_3$ ), which is carbon dioxide in solution, while the alkaline factor is chiefly bicarbonate:  $\text{NaHCO}_3$  in the blood plasma,  $\text{KHCO}_3$  in the corpuscles or  $\text{BHCO}_3$  for both. VAN SLYKE (see PETERS & VAN SLYKE, 1931) emphasized the importance of the bicarbonates and developed the idea that they are the "alkaline reserve" of the blood that protects the blood from acidity. He recognized, that in order to determine the alkaline reserve, one needed only to measure the amount of carbon dioxide that could be obtained from a sample of blood.

This new conception was soon adopted to fit the theory of an acidotic change in the blood as the cause of the combination of hypocapnia and hypocarbia that, so often, prevails in conditions of oxygen deficiency.

Mainly as the result of investigations by HALDANE, KELLAS & KENNAWAY (1919) and Y. HENDERSON & HAGGARD (1919—20) this theory of "asphyxial acidosis" was, however, shown to be a fallacy. These investigators developed the now generally accepted conception, according to which the hypocarbia in oxygen deficiency is not a primary change, but the result of a compensatory mechanism. As will be clear from the following pages evidence points to the combination of hypocapnia and hypocarbia in oxygen deficiency being the result of a compensated alkalosis and mainly of a non-acidotic nature.

### General Classification of Acid-Base Displacements in the Blood.

It is now well recognized that the acid-base balance of the blood can be expressed in terms of three variables, viz. carbon dioxide pressure, bicarbonate concentration and hydrogen ion concentration. It is also well known that the reaction of the blood is normally to the alkaline side of the neutral point, showing only slight deviations under normal, as well as under tolerated pathological or experimental conditions. The relationships between the three variables are conveniently expressed in conformity with the HENDERSON-HASSELBALCH equation:

$$[\text{H}] = K \times \frac{[\text{H}_2\text{CO}_3]}{[\text{BHCO}_3]}. \quad [\text{H}] \text{ expresses the hydrogen ion concentration}$$

of the blood;  $K$  is a constant;  $[\text{BHCO}_3]$  is the bicarbonate concentration of the blood and  $[\text{H}_2\text{CO}_3]$  is the amount of carbon dioxide in the form of carbonic acid in physical solution. From this equation one variable is fixed by simultaneous determinations of any two of the others. The physiological and clinical significance of the direction and extent in displacement of these variables was first pointed out by VAN SLYKE (1921), and the matter has later been thoroughly treated by PETERS & VAN SLYKE (1931).

Starting from normal conditions, deviation from the acid-base balance may be classified into two general types: a) *respiratory*, due primarily to carbon dioxide excess or deficit and b) *metabolic*, due primarily to fixed alkali excess or deficit without respiratory complications. Thus four major paths of displacement in the acid-base balance are primarily possible, the directions of which are towards

1) respiratory acidosis, 2) respiratory alkalosis, 3) metabolic alkalosis and 4) metabolic acidosis.

This classification was first suggested by PETERS & VAN SLYKE (1931) and was later supported by SHOCK & HASTINGS (1935). It also involves conditions of combinations between the four primary changes and conditions in which there is only insignificant deviation of the reaction towards acidity or alkalinity, although the carbon dioxide pressure and bicarbonate concentration is greatly changed, viz. compensated acidosis and alkalosis.

As will be clear from the following review, the changes in the acid-base balance of the plasma in oxygen deficiency are characterized either by hypocapnia solely (respiratory alkalosis) or by a combination of hypocapnia and hypocarbia. Studies by many investigators of the changes in the acid-base balance of the plasma during oxygen deficiency have led to conflicting views as to whether this combination is due mainly to a metabolic acidosis or to a compensated respiratory alkalosis.

### Hypocapnia.

It is well known that under normal conditions, excluding heavy work, the reactions of the human organism are directed so as to maintain, by alterations in the pulmonary ventilation, a constant partial pressure of carbon dioxide in the alveoli, and therefore in the arterial blood (about 40 mm Hg). This was first pointed out by HALDANE & PRIESTLEY (1905). It is also well known that, if to any degree the partial oxygen pressure in the inspired air is diminished, the arterial carbon dioxide pressure will also decrease. The loss of carbon dioxide from the body at high altitudes was first described by Mosso (1904), who termed this condition "acapnia". Although his finding was of primary importance for subsequent altitude research, his idea, that as a purely physical consequence of the lowered atmospheric pressure more carbon dioxide than usual is washed out of the blood in the lungs, was erroneous. BOYCOTT & HALDANE (1908) clearly demonstrated that the excessive loss of carbon dioxide under diminished atmospheric pressure is entirely due to oxygen deficiency. It was completely absent if oxygen was added to the air during decompression.

It is now firmly established that the direct cause of decreased arterial carbon dioxide pressure (hypocapnia) in oxygen deficiency is the increase in pulmonary ventilation. The hypoxic hyperventilation immediately causes a decrease of the carbon dioxide in physical solution — or carbonic acid — in the blood by lowering

the alveolar carbon dioxide pressure. For complete reviews see HALDANE (1935), NIELSEN (1936), and Y. HENDERSON (1938).

The general character of the decrease in alveolar and arterial carbon dioxide pressure under the influence of oxygen deficiency in the inspired air has been the object of painstaking determinations in decompression chambers and during special altitude expeditions. Notable examples of the latter are the parties organized by HALDANE (Pike's Peak, Colorado, 1911), BARCROFT (Peruvian Andes, 1921—22) and KEYS (International Altitude Expedition to Chile, 1935). The effect upon the carbon dioxide pressure in the alveoli of a rapidly induced, constant degree of oxygen deficiency in the inspired air was particularly investigated by BOYCOTT & HALDANE (1908), WARD (1908), DOUGLAS, HALDANE, HENDERSON & SCHNEIDER (1913), HASSELBALCH & LINDHARD (1915), NIELSEN (1936) and others. By summing up these observations the following characteristics of hypoxic hypocapnia are obtained. The hypocapnic state at any given degree of tolerated oxygen deficiency in the inspired air at rest does not reach its full development immediately, but gradually attains a final equilibrium with an individual rapidity. The time it takes for the full development varies from some hours to days or even weeks. The onset of the lowering is, however, rapid, so that by far the greatest part of the lowering is accomplished within a few hours or less.

Very careful observations have been made by FITZGERALD (1914) bearing upon the relations between the barometric pressure of many altitudes and the alveolar pressures of carbon dioxide of their acclimatized inhabitants. Her observations cover the range from sea-level up to 11,000 feet, and apply to human individuals, accustomed to different climates, diets and other conditions. It is remarkable that in the diagram, which she has given to express the relation of alveolar carbon dioxide pressures to the barometric pressure, the proportion is near to simple linear. She found a decrease of 4.2 mm Hg of alveolar carbon dioxide pressure per 100 mm Hg decrease in barometric pressure. With the data from the Pike's Peak expedition the series is extended up to 14,000 feet. From experiments with prolonged exposures to oxygen deficiency in a decompression chamber HASSELBALCH & LINDHARD found the same linear relationship between the decrease in alveolar carbon dioxide pressure and ambient barometric pressure.



From the above it is clear that if the bicarbonates in the plasma were constant during hypoxic hyperventilation, the hypocapnia must run parallel with alkalosis, *i. e.* a condition of respiratory alkalosis should be set up and maintained. For instance, at an ambient oxygen pressure corresponding to 10 % of an atmosphere, the arterial carbon dioxide pressure will gradually be decreased to a value of about 20 mm Hg. If the sea-level value be 40 mm this decrease in carbon dioxide pressure means that the carbonic acid/bicarbonate ratio should be halved. Consequently the  $cH$  of the plasma is also halved, *i. e.* the pH of the plasma is increased by  $\log 2$ , or approximately 0.3. It will, however, be seen below that the bicarbonate concentration of the plasma does not remain constant during prolonged exposure to oxygen deficiency, but slowly decreases so that the alkalosis is gradually compensated.

### Hypocarbia.

It has for a long time been known that the carbon dioxide content of the blood, *i. e.* the amount in physical solution and chemical combination, diminishes during residence at high altitudes. The decrease of the "titration alkalinity" of the blood as a result of oxygen deficiency was first demonstrated by GALEOTTI (1904). It was confirmed by the data from the expedition to the Peruvian Andes (BARCROFT et al., 1922). For complete references see BARCROFT (1925), HALDANE (1927), LILJESTRAND (1928), HALDANE & PRIESTLEY (1935), NIELSEN (1936) and Y. HENDERSON (1938).

The decrease of the bicarbonate concentration of the plasma (hypocarbia), and the consequent decrease in its titration alkalinity, was first thought to be a real change of the plasma reaction to increased acidity. It is now known, however, that the pH of the blood will return to normal during the process of acclimatization to oxygen deficiency (*cf.* next section). With the modern conception of the main factors in the acid-base balance of the blood it may therefore be inferred, that in the acclimatized condition there must be a definite relation of the plasma bicarbonates to the barometric pressure or, more exactly, to the ambient oxygen partial pressure. This follows from FITZGERALD's finding of the lowering of the alveolar carbon dioxide with the barometric pressure and from the HENDERSON-HASSELBALCH equation.

It was, however, not until comparatively late that the modern conception of the nature of the hypocarbic change in the blood in oxygen deficiency was arrived at. It was done independently by HALDANE, KELLAS & KENNAWAY (1919) and Y. HENDERSON & HAGGARD (1919—20). HALDANE, KELLAS & KENNAWAY found that during the initial stages of hypoxic hyperventilation the excretion of acid and formation of ammonia by the kidneys are greatly diminished, and the excretion of alkali is increased. They concluded that the hypocarbic change in the blood in oxygen deficiency did not consist in the production of a slight acidosis, but in the compensation of an alkalosis by the quite normal adaptive response of diminished excretion of acid and formation of ammonia. Y. HENDERSON & HAGGARD approached the matter from the same point of view. They found — as had been done before — that, in acute oxygen lack, respiration was increased with a corresponding lowering of the alveolar carbon dioxide pressure. But they also found that the carbon dioxide-combining power of the blood, which is directly dependent on the alkali in use in the plasma, was unchanged. This must mean a shift to alkalinity in the plasma. The same authors also made experiments on dogs, where oxygen deficiency was slowly induced during the course of several hours. They were able to demonstrate that the hypocapnic state in the blood is followed by hypocarbia, compensating more or less the hypocapnia. A few years earlier HENDERSON & HAGGARD had shown, that by merely varying the ventilation of the lungs and thereby adjusting the carbon dioxide pressure they could induce a marked decrease or increase in the alkali in use as bicarbonates in the plasma. When the lungs were overventilated, and the carbon dioxide in the blood was thus decreased, the bicarbonates also were greatly diminished. HENDERSON & HAGGARD pointed out that since, when a state of acute alkalosis is produced by forced breathing, the available alkali is greatly diminished in the plasma, the same thing must occur in the blood during the increased breathing caused by oxygen want. The alkalosis should lead to the disappearance of alkali from the plasma, also when there is shortage of oxygen. The authors assumed a migration of alkali from the plasma into the corpuscles, tissues and urine (see HENDERSON, 1938).

Concerning the possibility of metabolic acidosis, existing as a minor component in the hypocarbia of oxygen deficiency at rest, further investigation is indicated. The implication of metabolic

acidosis has been pursued by KAMEI (1931), THIEL (1933) and HASHIMOTO (1936). They argue that, since the alkali and pH of the blood are low and the lactic acid concentration high in the last stage of asphyxia, there must be blood acidosis. To this interpretation it may be objected, that in intense hypoxemia respiration is often depressed, carbon dioxide accumulates, and the  $\text{H}_2\text{CO}_3/\text{BHCO}_3$  ratio swings to the acid side. Hence, this acidosis is primarily of the respiratory type. As to the findings of increased lactic acid in the blood this is in perfect harmony with the fact revealed by GESELL, KREUGER, GORHAM & BERNTHAL (1930), that in cases where the hyperventilation from oxygen deficiency is artificially hampered, considerable lactacidemia will develop. As yet no evidence exists for an assumption, that in tolerated oxygen deficiency at rest with respiratory compensation intact a significant component of metabolic acidosis is prevailing. Investigations of the influence of rarefied air at altitudes and in decompression chambers (RYFFEL 1909, HARTMANN & V. MURALT 1934, EDWARDS 1936, NIELSEN 1936 and DILL, ABBOT & CONSOLARIO 1937) have shown no increase, but in some cases an actual decrease in the production of lactic acid at rest.

The development of the hypocarbic state in the plasma during the influence of a constant degree of oxygen lack in the inspired air is definitely more slow than the decrease in carbon dioxide pressure. Like the arterial carbon dioxide pressure the bicarbonate concentration approaches a final value, approximately directly proportional to the oxygen pressure in the inspired air (for references see PETERS & V. SLYKE 1931 and Y. HENDERSON 1938). The decrease is delayed so there is a definite discrepancy between the rates in decrease of carbon dioxide pressure and bicarbonate concentration in the acute stage of oxygen deficiency.

### The Hydrogen Ion Concentration.

From the above it may be realized, in conformity with the conclusions now generally accepted, that in the initial stages of tolerated oxygen deficiency there is a respiratory alkalosis, which may be very marked. The degree of alkalosis is in the main determined by the actual values of the nominator and denominator of the HENDERSON-HASSELBALCH equation. The respiratory alkalosis is gradually compensated by a decrease of the alkali in use in the plasma. It remains to be proved if to any

degree a component of metabolic acidosis is also present. As yet no definite conclusions can be made concerning the rapidity, with which the alkalosis develops and subsides at rest during a given degree of oxygen deficiency in the inspired air, but it seems that the alkalosis attains its maximum within the first hours of hypoxic hyperventilation.

Direct recordings by means of modern electrometric methods have confirmed indirect calculation. The alkalosis of acute oxygen deficiency was first demonstrated electrometrically in the rabbit by WINTERSTEIN (1915). His finding led to a revision of the first Winterstein "reaction theory" (cf. below). HASSELNACH & LINDHARD were the first to show electrometrically (1915), that after prolonged exposure to oxygen deficiency there is no significant change in the pH of the blood. These early findings have been sufficiently confirmed by subsequent investigators. The swing to alkalinity may cover some tenths of a pH during the first hour of hypoxic hyperventilation (BRASSFIELD, 1936). For references see WITTKOWER (1933) and NIELSEN (1936).

Repeated recordings of blood pH during prolonged exposure to a given degree of oxygen deficiency seem to have been made only by NIELSEN (1936). He found in two subjects at a barometric pressure of 437 mm an increased pH, gradually returning to normal within the course of some days. The alkalosis was very slight, however, and no values were recorded within the important first hours of reduced oxygen pressure.

An insignificant part of the alkaline change in the blood in hypoxemia is caused directly by the reduction of oxyhemoglobin, for fully oxygenated blood is more acid than reduced blood at a given carbon dioxide pressure (PARSONS, 1917). Accordingly, GOLLWITZER-MEIER (1942) found an alkaline change of  $\text{pH} + 0.029$  in a dog that was subjected to 6.4 per cent oxygen after the chemoreceptors had been denervated. The pulmonary ventilation was actually decreased by the influence of oxygen deficiency upon the chemoreceptively denervated center.

## 2. The Paradox of Hypoxic Hyperventilation.

Some theories on the chemical mechanism of hypoxic hyperventilation have necessarily already been touched upon in the preceding section. In the following a more continuous account will be given of this subject, which still is very incompletely under-

stood. The extensive research activities, which have so far contributed to our knowledge of the chemical control of breathing in oxygen deficiency, can readily be divided into two epochs, when the consequences of the discovery of the peripheral chemoreceptor mechanism are taken into consideration. It is probably safe to say that no physiologist, working on this subject, had actually been able to demonstrate that oxygen lack, restricted solely to the centers, will produce increased ventilation. Yet, until the observations of C. HEYMANS and his associates on the peripheral chemoreflex mechanism, indirect evidence seemed to confirm the general conviction that the chemical control of respiration under oxygen lack resided solely in the respiratory center. However, in the light of present evidence, we now know that physiologists at that time worked upon an uncertain foundation, when taking such a monopoly of the respiratory center as granted. So when HEYMANS in 1938 was awarded the Nobel prize in Physiology he was paid an official tribute for a series of works, which has practically commenced a new era in the conception of the chemical control of respiration.

Today it may invite wonder as to how the hyperventilation in acute oxygen deficiency was explained at a time when nothing was known about the dominating rôle of the chemoreflexes. It is true that the current theories on this subject suffered in prestige during the temporary confusion that followed the discovery of the chemoreflex mechanism. However, some previous works on this subject, at the time applying to the respiratory center solely, are in fact as valid today, even if the existence of the chemoreflexes could not be anticipated. The theories advanced in these works actually applied to the entire respiratory chemosensitive apparatus, and will in the following review be treated accordingly.

### The Time before the Discovery of the Chemoreflex Mechanism.

At the time, when the BOYCOTT & HALDANE theory (1908) of lactic acid acidosis as the extra stimulus to the respiratory activity in oxygen deficiency was advanced, few conceptions in the range of physiology appeared more certain than that, if to any degree the supply of oxygen falls short of the demand, lactic acid must accumulate in the blood and induce a corresponding degree of blood acidosis. In the light of the knowledge of the

respiratory response to increased blood acidity the hypoxic hyperpnea therefore was no paradox. The conception of "asphyxial acidosis", based upon the early work of Araki, was extended to apply to even moderate degrees of oxygen lack. Also in the first "reaction theory" of WINTERSTEIN (1911) this conception was employed for an explanation of the hypoxic hyperventilation. The respiratory activity was meant to be controlled by the reaction of the blood.

The "fallacy of asphyxial acidosis" (HAGGARD & HENDERSON 1919—1920, HENDERSON 1938), however, soon became undermined by the lack of experimental evidence. From the preceding section it is now clear 1) that there is no increased production of acid involved in tolerated oxygen deficiency at rest and 2) that in acute hypoxic hyperventilation there is a definite swing to alkalinity in the blood.

Experimental evidence against an acidotic mechanism began with the discovery of RYFFEL (1909) that there is no appreciable excess of lactic acid in the blood of persons exposed to quite low pressures of oxygen, and the finding of HASSELDALCH & LINDHARD (1915), that there was no significant decrease of the blood pH after prolonged exposures to oxygen deficiency, in spite of the prevailing hyperventilation and in spite of a great diminution in the titration alkalinity of the blood.

From this time on the hypoxic hyperventilation became a real paradox. The gradually accumulating evidence in favour of an alkaline shift in the blood during the hyperventilation of acute oxygen deficiency seemed definitely contradictory to the well known response of the respiratory apparatus to increased blood acidity.

WINTERSTEIN, who soon also found increased alkalinity of the blood during hypoxic hyperventilation (1915), therefore advanced a second "reaction theory" (1921), according to which the respiratory activity should be controlled by the reaction within the respiratory center itself. In oxygen deficiency there should be a local acidosis in the respiratory center, induced by increased local production of lactic acid.

WINTERSTEIN's theory on the "centrogenic regulation of respiration" under the influence of oxygen deficiency has been vigorously supported by the GESELL School. In 1923 GESELL adopted this theory by stating that the activity of the respiratory center is fundamentally a function of its own intracellular acidity as opposed to the reaction of the arterial blood, the specificity of

carbon dioxide and the direct stimulating action of lack of oxygen (cf. GESELL 1939).

In its original form the cellular acidity theory is open to criticism. The requirement of an increase of the intracellular acidity within the center itself during the hyperventilation of acute and moderate oxygen deficiency has not met with sufficient experimental support. In fact, strong arguments against the application of this theory to the center present themselves in recent findings of GESELL himself (GESELL, LAPIDES & LEVIN, 1940), although this particular conclusion was not drawn. By cold-blocking the sinus nerves during acute hypoxic hyperventilation after bilateral vagotomy in dogs, all known afferent chemosensory impulses to the center were cut off. Thus the basal inherent activity of the center itself, free from the chemoreflex drive, could be demonstrated. It was clearly shown that the centrogenic component during acute hypoxic hyperventilation is definitely less than during eupnea.

From these blocking experiments it can be inferred that the chemosensitive cells of the center itself have no direct share in the hyperventilation of acute oxygen deficiency. The influence upon the chemosensitive cells of the center of changes in arterial pH and carbon dioxide pressure was, however, clearly demonstrated by these experiments, for it seems most probable that the decrease of the centrogenic component was due to the increasingly hypocapnic and alkaline state of the blood.

Different kinds of direct experimental approach to the question of intracellular lactic acid acidosis in hypoxia with respect to the respiratory center itself, were made in the works of MCGINTY & GESELL (1925) and MCGINTY (1929) on the effects of cerebral anemia, cyanidemia, hypoxemia, and hemorrhage on the lactic acid content of the brain. In the former work the lactic acid content was investigated at varying intervals after decapitation and complete circulatory isolation of the brain. The finding of increased lactic acid was interpreted as supporting the intracellular acidity theory. A similar conclusion was drawn from the results of the latter work, demonstrating increased lactic acid content in the venous blood from the brain, in conditions in which the arteries to the brain were partly or completely occluded, in which cyanide poisoning was induced, and in which pulmonary ventilation was depressed. It is, however, highly questionable if these works contribute to the validity of the intracellular acidity

theory, as has emphatically been maintained. To the above interpretation of the results it can legitimately be objected, that the conditions induced can hardly be compared to the influence of tolerated oxygen deficiency upon the cerebral tissue of living intact animals with respiratory and circulatory compensating mechanisms at high vitality.

Moreover, direct recordings by means of the glass electrode of the pH of the surface and interior of the brain, including the center, has revealed that the brain as a whole becomes more alkaline than normal during acute oxygen deficiency (GELLHORN & LAMBERT 1939, INGRAHAM & GELLHORN 1939).

There is thus less reason now to cling to the old hypothesis of increased intracellular acidity of the respiratory center itself as the cause of even part of the increased respiratory activity of the center in acute tolerated oxygen deficiency at rest. The blocking experiments attest to that. The attempts to explain the paradox of hypoxic hyperventilation in terms of increased acidity within the respiratory center itself as opposed to increased blood alkalinity are, today, more applicable to the peripheral chemosensitive cells.

The required modification of the original cellular acidity theory seems to be foreshadowed by GSELL in one of his latest reviews (1941). To quote: "The cellular acidity theory demands that only those structures designed to correct against oxygen lack withstand the impact of oxygen shortage. — — — As it has finally turned out, the tiny collections of chemoreceptors found in the carotid and aortic bodies are the regulators of breathing against oxygen lack. They alone stand the brunt of impaired oxidations and by their activity protect the body as a whole. As we shall show, the respiratory center actually becomes asapnic with anoxic hyperpnea for when HERING's nerves are blocked breathing stops. But this does not imply that the center is excluded in the general scheme of the cellular acid theory of respiratory control. Like the chemoreceptors it responds to changes in acidity."

It will be shown (see below: "The rôle of the chemoreflex mechanism") that there is now actual experimental evidence for an intracellular acid excitation of the chemoreceptors as opposed to the alkaline change in the blood during acute hypoxemia.

The works of HAGGARD & HENDERSON in 1919—1920 on "Hemato-respiratory functions" largely contributed to revealing



the nature of the change in the acid-base balance during oxygen deficiency of varying durations of exposure. Y. HENDERSON was at the time one of the chief opposers of the implication of acidosis in oxygen deficiency. Together with HAGGARD he developed a theory of "respiratory X" as the cause of the hypoxic hyperventilation. About the nature of this mysterious agent nothing could be said, except that it was not a strong, fixed acid. "Respiratory X" should not merely be the result of reduced oxygen, but rather mainly the resultant of the ambient oxygen pressure and the actual alkali content of the blood. Whenever the  $pO_2$ /alkali ratio is below normal the amount of respiratory X should be increased. As a direct result overbreathing should occur, causing an excessive blowing off of carbon dioxide and a simultaneous decrease of the carbon dioxide/bicarbonate ratio in the plasma. As the alkali passes out of the blood, the  $pO_2$ /alkali as well as the carbon dioxide/bicarbonate ratios should gradually return to normal.

The theory of HAGGARD & HENDERSON is interesting because the characteristic features of prolonged exposure to oxygen deficiency are outstandingly integrant. It is probably safe to say, that no other hitherto advanced theory on the nature of the chemical stimulus to respiration during oxygen lack has been complete in the sense that the mechanism of prolonged hypoxic hyperventilation has been taken into special consideration.

If we understand HENDERSON correctly his conception of "respiratory X" can be transferred into the very component in the resultant hyperventilation, for which the oxygen lack is responsible. The remaining component may be regarded as the need for removal of carbon dioxide. Hence, the paradox of acute hypoxic hyperventilation with hypocapnia and increased blood alkalinity was explained by an additional component of unknown character. This component should be directly proportional to the blood alkali/ $pO_2$  ratio. As the process of acclimatization to the ambient oxygen pressure advances, the alkali in the circulating blood decreases, and the respiratory X component will thus decrease in proportion to the alkali. When acclimatization is complete the blood alkali/ $pO_2$  ratio and consequently the respiratory X component has subsided to its eupneic value. After acclimatization is established the continuous hyperventilation is therefore mainly controlled by the need for carbon dioxide removal, as is the breathing under normal oxygen pressure.

A point of fundamental importance for the reasoning was the fact that metabolism is largely independent of variations in tolerated ambient oxygen pressures, as had been found by DOUGLAS on Pike's Peak and by HASSELBALCH & LINDHARD in decompression chamber experiments. The amount of carbon dioxide produced during rest at any tolerated altitude is therefore the same as during rest at sea level. It follows logically, that as the bicarbonate concentration of the plasma *i. e.* its buffering capacity, is decreased after prolonged exposure to low oxygen, the ventilation must increase if the plasma pH shall remain constant. Hence an increased ventilation must be maintained by the need for carbon dioxide removal.

Although the respiratory X theory has now lost a great deal of its former authority because of the discovery of the chemoreflex control of the hyperventilation of acute oxygen deficiency, the author has given a relatively complete account of it here for the following reason. The theory yields a very important conclusion, based upon a logical reasoning: the paradoxical discrepancy between the amount of respiratory activity and the actual state of the acid-base balance of the blood seems to decrease and finally disappear, if the exposure to a given degree of oxygen deficiency is sufficiently prolonged.

HENDERSON advanced the idea, that respiration is mainly regulated by the carbon dioxide pressure of the blood, carbon dioxide acting not directly, but through its relation to the alkali in use in the blood. The paradox of acute hypoxic hyperventilation was explained as caused by an increased excitability of the respiratory apparatus to carbon dioxide. The increased excitability should be directly proportional to the actual amount of respiratory X (alkali in use/oxygen pressure ratio). Hence, after acclimatization to any tolerated altitude is accomplished, the excitability of the respiratory apparatus should be restored to normal, for the alkali bicarbonate concentration of the plasma gradually reaches a value, that corresponds to the ambient oxygen pressure.

A full account of the respiratory X theory is given by Y. HENDERSON in his book "Adventures in Respiration" (1938).

The intracellular acidity and the "extracellular" respiratory X theories have met with pertinacious objection from several recent investigators, among which may be mentioned NIELSEN (1936) and SCHMIDT (1941, 1945). They maintain that the chemical

stimulus to the center under all circumstances is carbon dioxide acting, not as an acid, but through some specific property of its own. This opinion originates from the results of NIELSEN, demonstrating greater respiratory response to carbon dioxide inhalation than to ammonium chloride acidosis, although the blood reaction showed a greater shift to acidity in the second case than in the first. It is not the author's intention here to give a full critical consideration of this theory, especially as it is at present not quite clear, whether only the respiratory center itself or also the peripheral chemosensitive cells is considered. The original work of NIELSEN, however, certainly applied to the entire aggregate of central and peripheral chemosensitive cells, although the rôle of the latter was not accounted for. To his standpoint against the cellular acidity theory it might preliminarily be objected, that in each of the above-mentioned situations the change in blood reaction need not give a quantitative indication of the intracellular reaction of the center or the chemoreceptors. The findings of NIELSEN, therefore, give no argument against the intracellular theory, either the original or a modified one. Apart from their failure to give any arguments against the intracellular acidity theory, these findings are not contradictory even to a conception of carbon dioxide exerting its action through the hydrogen ions of the blood in acidotic conditions, as NIELSEN, in fact, infers.

This is clearly intelligible, if one remembers that a conception of control through the hydrogen ions does not require that a certain pH shall induce a certain volume of breathing. The increased breathing in acidotic conditions may readily be explained in terms of hydrogen ion control by the fact that the primary change in the acid-base balance of the blood during metabolic acidosis is a decrease of the concentration of alkali bicarbonates. The consequently decreased buffering capacity of the blood against a given production of carbon dioxide must inevitably lead to increased pulmonary ventilation, if the hydrogen ion concentration shall remain constant, as it essentially does down to a very extensive reduction of the available alkali.

NIELSEN's implication of an increased excitability of the respiratory apparatus to carbon dioxide in acidotic conditions is, indeed, acceptable, but on a basis that is very definite and therefore more attractive, than the entirely hypothetical one that was used. For increased production of fixed acids reduces the amount

of buffering bicarbonate in the blood and tissues and thereby increases the excitability of the carbonic acid/bicarbonate ratio, and consequently that of the  $cH$ , to a given change of the carbon dioxide pressure. Granting the respiratory apparatus to be sensitive to changes in the  $cH$  of the blood, we have here a conception of increased excitability of the respiratory apparatus to carbon dioxide in hypocarbic conditions on a quite logical basis.

Concerning the paradox of hypoxic hyperventilation, it would seem reasonable that the effectiveness of carbon dioxide would be in proportion to its molecular concentration. This requirement is not easily fulfilled, if one considers the paradoxical hypocapnic state in the blood and center. NIELSEN therefore proposed the accessory theory — as did Y. HENDERSON — that in oxygen deficiency the excitability of the respiratory apparatus ("center") should be increased, employing the original postulate of LINDHARD (1911). LINDHARD made, however, no distinction between carbon dioxide and acidity as the stimulus, as did NIELSEN. NIELSEN gave no clue to how such an increased excitability to carbon dioxide during oxygen lack should be explained, apart from an implication, that it might be caused by a reflex influence from the peripheral chemoreceptors. Having interpreted his experiments on acidosis in the above-mentioned manner, he generalized his idea on the specific action of carbon dioxide obtained from these experiments to apply also to the condition of oxygen deficiency. By referring only to the carbon dioxide pressure, the theory was meant to apply also to prolonged conditions of oxygen deficiency by assuming the excitability to be continuously enhanced (cf. the respiratory X theory).

The theory of the specific action of carbon dioxide, which in fact means a conception of respiratory regulation by one of the variables in the acid-base balance of the blood, attaches only minor importance to an influence of the other variables, viz. the bicarbonate concentration and the  $cH$ . To the author this seems to be inconsistent with another feature of the postulate, i. e. the one that the excitability of the respiratory apparatus should be increased in conditions of metabolic acidosis. For after prolonged exposure to oxygen deficiency the acid-base condition of the blood is much the same as in metabolic acidosis, although fundamentally a compensated respiratory alkalosis. The NIELSEN theory actually demands the implication of twofold excitability increase after prolonged exposure to oxygen deficiency, for the

excitability of the respiratory apparatus to a given increase of carbon dioxide pressure is also increased as a result of the decrease of the buffering capacity of the blood. Such a mechanism in the "chronic" stage of oxygen deficiency was, however, not proposed.

The HENDERSON and NIELSEN theories of extracellular chemical control of the acute hypoxic hyperventilation both include a conception of increased excitability as a central integrant. To the author there seems to be full reason to regard the activity of the respiratory apparatus as the product of at least two main factors: 1) the strength of the stimulus, and 2) the sensitivity, or excitability, of the respiratory apparatus to the stimulus. There should not be anything surprising in the assumption that there may be changes in the sensitivity of the respiratory apparatus to carbon dioxide or  $\text{cH}$  under certain abnormal conditions, as during general anaesthesia and in other conditions in which the vitality of the respiratory apparatus is changed. In acute oxygen deficiency there are, however, at least three possibilities concerning the cause of the increased activity of the respiratory apparatus: 1) increased excitability, 2) increased excitation, and 3) a combination of 1) and 2). Only the first possibility was discussed by HENDERSON and NIELSEN. The author knows of no actual experimental proof for the postulate that in acute oxygen deficiency the excitability of the respiratory apparatus should be increased. The arguments which have been given in favour of this belief are all applicable for a theory of increased excitation as well. In view of the present knowledge of chemoreflex control a conception of increased excitation actually seems more acceptable. It might seem that the question of increased excitation contra excitability is a wordy warfare. However, the theory of increased excitability is not quite acceptable as long as experimental evidence is lacking. In the special conditions, where the buffering capacity of the blood against carbon dioxide is diminished, as after prolonged exposure to oxygen deficiency (compensated respiratory alkalosis) or in conditions of metabolic acidosis, there is a logic reason for an assumption of increased excitability of the respiratory apparatus to carbon dioxide. But, as has been pointed out before, this reason does not apply to the conception of the specificity of carbon dioxide.

## The Rôle of the Chemoreflexes.

In the preceding pages an account has been given of the most important theories through which the apparent contradiction of hypocapnia (alkalosis) and hyperventilation in acute oxygen deficiency has been explained. Granted certain modifications, these theories can be regarded as being as valid to-day as they were at the time when the existence of the chemoreflexes was not known. After all, the receptors in the carotid and aortic bodies are a part of the neuro-respiratory mechanism, that was formerly denominated as the respiratory center. From the teleological point of view one may ask why these structures, which are so specifically sensitive to hypoxemia, are situated so far from the other chemosensitive cells of the respiratory apparatus. Perhaps the reflex character of the connections between these sets of chemosensitive cells is significant. As will be shown, certain arguments for such an assumption are obtained from the experiments of the present work.

A number of different workers have confirmed in main the results of J. F. and C. HEYMANS and C. HEYMANS and his co-workers, obtained in the years 1924—27 and 1930—32. If one considers that the subject of peripheral control has developed in less than fifteen years it is, however, not surprising that the significance of chemoreflex activity has not yet been fully elucidated. Significant in this respect is the fact, that the rôle of the chemoreflexes for even moderately prolonged hypoxic hyperventilation has not been inquired into and still less investigated. That there is full reason for such an investigation will clearly be shown.

The dominating rôle of the chemoreflexes for the hyperventilation of acute oxygen deficiency is firmly established. For reviews see HEYMANS, BOUCKAERT & REGNIERS (1933), GESELL (1939, 1941), SCHMIDT & COMROE (1941), BERNTHAL (1944) and SCHMIDT (1945).

From the fact that the chemoreflexes have been shown to be entirely responsible for the increased breathing in acute oxygen deficiency and from the fact that hypereapnia and increased acidity of the blood have been shown to be powerful stimulants to the chemoreceptors, it is obvious that the paradoxical hypocapnia (alkalosis) in acute hypoxic hyperventilation is especially striking with respect to the increased activity of the chemoreceptors.

Some fundamental features of the chemoreceptor activity and the chemoreflex drive in acute oxygen deficiency will be summarized in the following pages.

### *a) Chemoreflex Thresholds in Hypoxemia.*

The most direct evidence of chemoreceptor activity under high arterial oxygen saturation has been obtained by studies on action potentials in the sinus nerve. This method was employed by EULER, LILJESTRAND & ZOTTERMAN (1939), who found an increase of the discharge in the chemoceptive fibres as soon as the arterial oxygen saturation fell below 96 per cent. The response might have begun earlier, had not a certain degree of hypocapnia been induced by artificial overventilation. This most direct criterion of chemoreceptor activity has not been approved by SCHMIDT & COMROE (1941), who argue that the electrical activity may have originated elsewhere. It is, however, difficult to understand why these potentials should disappear upon hyperventilation with oxygen and increase as a response to hypoxemia and hypercapnia, if they were not action potentials in chemosensory fibres. This has recently been pointed out by BERNTHAL (1944), and GESELL (1941) has also adopted the original interpretation of EULER, LILJESTRAND & ZOTTERMAN. In view of the results of recent investigations performed by the same authors with identical technique (EULER, LILJESTRAND & ZOTTERMAN 1941, EULER & ZOTTERMAN 1942) the argument of SCHMIDT & COMROE cannot possibly survive.

The firing frequency was also recorded under diminished oxygen saturation of the blood supplying the carotid body. The carbon dioxide pressure was thereby continuously kept below its exciting threshold, *e. g.* 30 mm Hg., by means of constant artificial overventilation. The relation between the increase of potential frequency to oxygen unsaturation was found to be near to linear down to 52 per cent. Of course, this finding does not mean that a linear increase of the impulse frequency in the sinus nerves will bring about a linear increase of the activity of the center under all circumstances, or even that any given firing activity will be responsible for part of the respiratory activity. It is very probable (*cf.* below) that the response of the center to afferent impulses is greatly influenced by changes in the acid-base balance in the blood. Chemoreceptor disconnection affords another possibility of judging whether chemoreceptor activity is prevailing under eupneic activities of the respiratory center. SELLADURAI & WRIGHT (1932) and EULER & LILJESTRAND (1936, 1940) have observed that denervation causes a decrease in ventilation in animals un-

der normal experimental conditions. The latter authors were able to show a marked increase of resting alveolar carbon dioxide pressure, even if the animals, dogs and cats, were breathing oxygen. The experiments prove that, under anaesthesia, a continuous discharge of impulses is sent out by the chemoreceptors, causing an increase of the respiratory activity.

Certain conclusions as to the existence of chemoreceptor activity under eupneic hypoxemia may also be drawn from experiments with inhalation of air enriched in oxygen content. EULER & LILJESTRAND (1942) have found, that oxygen substituted for air in anaesthetized cats or dogs causes an almost instantaneous, sometimes very considerable, reduction of respiratory rate and amplitude. This effect was abolished by denervation of both sinuses and was thus due to the disappearance of a slight oxygen want which stimulated the chemoreceptors of the carotid body. After some minutes of oxygen breathing the ventilation again closely approximated to the value from air breathing. This compensation was partly explained by an increased carbon dioxide pressure in the arterial blood. Similar results were obtained by WATT, DUMKE & COMROE (1942—43) upon normal unanaesthetized dogs. Oxygen inhalation, which removed all hypoxemic stimulation of chemoreceptors, led to a decrease of the ventilation, varying from 11 to 31 per cent, and the authors concluded as did EULER & LILJESTRAND that this must be the result of the withdrawal of a chemoreflex drive, elicited by the usual degree of oxygen unsaturation of the arterial blood at sea-level.

From the above it can be inferred that the chemoreceptor threshold for diminished oxygen pressure must be placed at some level higher than that of eupneic arterial blood.

*b) The Nature of the Chemical Stimulus to the Chemoreceptors in Hypoxemia.*

A tonic activity of the chemoreflex mechanism, responsible for an appreciable part of the activity of the respiratory center at eupneic levels of arterial oxygen pressure is thus firmly established with respect to animals.

It is now pertinent to question if the chemoreceptor activity in eupnea and in acute hypoxic hyperpnea is set up by different or by the same chemical disturbances in the chemoreceptors. In acute hypoxic hyperpnea those physiological stimuli, which are



known to stimulate the chemoreceptors, viz. carbon dioxide and hydrogen ions, are decreased and mainly the stimulating effect of oxygen deficiency remains. However, evidence points to a combination of hypoxemic and hypercapnic stimulation of the carotid bodies in eupnea (SAMAAN & STELLA 1935, EULER & LILJESTRAND 1936, BERNTHAL 1938, EULER, LILJESTRAND & ZOTTERMAN 1939 and WINDER 1942).

Indirect as well as direct evidence has permitted the assumption, that the exciting factor to the chemoreceptors is a change of the hydrogen ion concentration to increased acidity within the chemoreceptive cells themselves (EULER, LILJESTRAND & ZOTTERMAN 1939). Especially when considering the resemblance in effect of intracellular hypoxia and hypercapnia — increased acidity being the common denominator — and bearing in mind that close injections of acid solutions have been shown to initiate strong firing from the chemoreceptors (ZOTTERMAN 1935), the introduction of changes in the hydrogen ion concentration in the receptive cells as the immediate controlling factor of excitation seems to afford a most convenient hypothesis. It has been directly tested by close injections of small amounts of ammonium hydroxide (1 ml of a solution of half normality). The result was a complete extinction of those impulses in the sinus nerve which were caused by hypoxemia or hypercapnia. The action of ammonium hydroxide was not poisonous, since the impulses soon reappeared, usually somewhat augmented.

The conception of intracellular hydrogen ion concentration as the immediate controlling factor of chemoreceptor excitation has not been accepted by COMROE & SCHMIDT (1938), who pointed out that hypoxemic blood may cause strong reflex hyperventilation although hypocapnic and alkaline, and that hypercapnic blood may be less active in arousing reflexes although more acid. They think it evident that lack of oxygen is the direct stimulus, and independent of acidity changes. Two important objections can, however, be raised against their interpretation of these well known facts. First, that no conclusions can be made concerning the relative chemoreceptor activity in hypoxemia and hypercapnia by judging from respiratory reactions. It has clearly been shown (cf. below) that in hypercapnic conditions the impulses from the chemoreceptors are effectively blocked by chemical modifications at the center, so that the chemoreflex drive is insignificant. But this is not meant to imply that the chemo-

receptor activity is also decreased. Therefore, chemoreflex drive is not the same as chemoreceptor activity. Another objection is that the employed facts do not constitute any proof against the intracellular acidity theory. The possibility of a changing relationship between intracellular and blood acidity seems to have been overlooked. Such a changing relationship between intracellular and extracellular hydrogen ion concentration was originally demonstrated by JACOBS (1920). He found that alkaline  $\text{H}_2\text{CO}_3\text{--NaHCO}_3$  solutions may be able to cause a shift to acidity within the cells of certain lower organisms and flowers. His finding is best known in its application to the second reaction theory of WINTERSTEIN (1921), which it seemed to support. Other experiments have been done, showing the tendency of undissociated carbon dioxide or carbonic acid from extracellular bicarbonate buffer mixtures to turn cells more acid, at least temporarily. However, the application of these findings is, as has been pointed out before, no longer necessary for the center itself in acute hypoxemia, but instead to the paradox of blood alkalinity and intracellular acid excitation of the chemoreceptors.

c) *The Response of the Chemoreceptively Denervated Center to Oxygen Lack.*

The ability of the chemosensitive cells of the respiratory center itself to respond with increased activity to oxygen lack has been investigated by different methods. Experiments, in which the central effect of hypoxemia was tested after the anaesthetized animal had been chemoreceptively denervated and after it had been breathing room air, were made by HEYMANS, BOUCKAERT & DAUTREBANDE (1930) on dogs, by SELLADURAI & WRIGHT (1932) on cats, by BEYNE, GAUTRELET & HALPERN (1933) on cats, by EULER & LILJESTRAND (1936) on dogs and cats, by GESELL & MOYER (1937) on dogs and by MOYER & BEECHER (1942) on dogs. Inhibition of respiration was reported throughout. However, MOYER & BEECHER working with only slightly anaesthetized animals found that after a latency period of some minutes a secondary stimulation would occur. The question is, however, whether the effect should be termed stimulation by oxygen lack or by accumulating carbon dioxide or acid metabolites. Significant in this respect was their finding of simultaneous signs of generalized motor stimulation.

The influence of narcotics upon the responsiveness of the center to oxygen lack has been investigated by using the technique of decerebration. In no case was stimulation observed (SELLADURAI & WRIGHT 1932, BOUCKAERT, HEYMANS & SAMAAAN 1938 and BOUCKAERT, GRIMSON & HEYMANS 1939). The effect of oxygen deficiency after chemoreceptive denervation has also been tested in the unanaesthetized state after recovery from the operation (DECHARNEUX 1934, HENDERSON & GREENBERG 1934, GEMMILL, GEILING & REEVES 1934, MARSHALL & ROSENFELD 1936, JONGBLOED 1936, SMYTH 1937, BOUCKAERT, HEYMANS & SAMAAAN 1938 and WATT, DUMKE & COMROE 1942—43). JONGBLOED, SMYTH and MARSHALL & ROSENFELD found only inhibition of respiration. GEMMILL, GEILING & REEVES found a response, but had not severed the aortic nerves. HENDERSON & GREENBERG, BOUCKAERT, HEYMANS & SAMAAAN and WATT, DUMKE & COMROE found only a late increase of the respiratory activity after a latency period of several minutes. As has been pointed out this delayed "stimulation" may be explained by gradual accumulation of carbon dioxide or acid metabolites as a consequence of the preceding insufficiency of breathing.

Experiments, in which the respiratory response was studied after cold-blocking of the chemoreceptive impulses during acute hypoxic hyperventilation (GESELL, LAPIDES & LEVIN 1940) have already been mentioned. They showed that in the anaesthetized condition withdrawal of the chemoreflex drive during hypoxic hyperventilation may cause immediate apnea. No "paralysis" of the center ensued, for it responded almost immediately on deblocking with a greater activity than before the block was applied, most probably due to rebuilding of the central stimulus (carbon dioxide or acidity) during the apneic period.

DECHARNEUX (1934) and DAUTREBANDE (1938) claim to have found perfect acclimatization to hypoxemia in unanaesthetized dogs, deprived in advance of the aortic and carotid chemoreceptors. This finding certainly points to a minor importance of the chemoreflex drive in prolonged oxygen deficiency, a point of special importance for the problem of the present work. There is, however, the possibility of two sources of error here, which must not be overlooked. The first one is regeneration of chemosensory fibres during the long interval between the denervation and the testing in the experiments of DECHARNEUX (a full account of DAUTREBANDE's experiments has not been available).

Such degeneration has been assumed by JOHNSON (1936). The second, also applying to the experiments of DECHAMPEUX, is that an altitude of only 7000 feet was tried. Actually, the arterial carbon dioxide pressure indicated no measurable increase of the ventilation, which was not directly recorded. It is therefore questionable if the experiment of DECHAMPEUX contributes at all to the important question of chemoreflex control of ventilation during prolonged oxygen deficiency. GESSALL (1939) states: "The possibility of an adaptive adjustment so common for nervous mechanisms, in this case a development of inherent potential sensitivity of the center to oxygen lack in the absence of normal peripheral support, has so far as we know not been considered. So firmly is the belief of central depression from anoxia established that a late positive response to oxygen lack in chronically denervated dogs is taken as proof of regeneration of chemosensory fibers. — Let us hope that DARTENAYAN will have the opportunity of determining in his dogs whether or not regeneration occurred." It will probably turn out to be an extremely difficult task to bring unreprouachable evidence concerning a central response to oxygen lack by such an experimental procedure since, even after a most painstaking denervation, not only remaining chemoreceptive reflexes but also gradually developing vicarious chemoreflexes would nullify the validity of the interpretation.

*d) Interaction of Chemoreflex and Centrogenic Control of Breathing in Acute Oxygen Deficiency.*

There can be no question whatever concerning the importance of the chemoreflexes for the hyperventilation of acute oxygen deficiency. The chemoreflex activity is due to an increased oxygen deficiency, and causes an excessive blowing off of carbon dioxide at a greater rate than corresponds to the actual formation. From the important findings of GESSALL, LAMDES & LEVIN (1940) it is obvious that the selfengendered activity of the chemosensitive cells of the respiratory center itself is actually decreased, and that the respiratory hyperactivity is entirely caused by the chemoreflex drive. It was found in the acute stage that the greater the oxygen deficiency the less is the centrogenic component. It seems very probable that the decrease of the basal activity of the chemosensitive cells of the center was due to the alkaline and hypocapnic change in the blood. In view of the assumed

similar intracellular change within the chemoreceptors in hypercapnia, one may ask how the activity of the chemoreceptors in hypercapnia cooperates with that of the chemosensitive cells of the center, which are known to be very sensitive to hypercapnia. Blocking experiments (GESELL, LAPIDES & LEVIN, 1940, cf. page 20) have shown that the centrogenic component in hypercapnia is the most important, and that the chemoreflex drive is actually decreased in strong hypercapnic hyperpnea. From this finding it may be realized that the activity of the chemoreceptors is without major influence upon the activity of the chemosensitive cells of the center in hypercapnic conditions. In experimental conditions, when the carotid bodies are separately perfused with hypercapnic solutions, the activity of the center is, however, also greatly increased, in spite of the hypocapnic and alkaline change in the blood supplying the center (HEYMANS & BOUCKAERT 1939). GESELL, LAPIDES & LEVIN (1940), concluded that the impulses along the afferent pathways in general hypercapnia are blocked by the hypercapnic state itself. Alternatively it may be inferred that when hypercapnia is induced solely at the chemoreceptors, the response of the center to the resulting firing in the sinus nerves is actually potentiated by the hypocapnic and alkaline change at the center. From a teleological point of view such an intermediary mechanism would explain why the chemosensitive cells so specifically reactive to hypoxemia should be connected to the remaining chemosensory cells of the respiratory apparatus by way of a reflex. For by adopting the assumption of chemical modification along the afferent pathway as an important factor in the chemoreflex drive of hypoxic hyperventilation, one has here a counter-balance to the restriction of the chemoreceptor activity which must necessarily follow as a result of the hypocapnic and alkaline change in the blood supplying the chemoreceptors.

In conclusion it may be said that available evidence seems to point to a general sensitivity of central and peripheral chemosensory cells to changes in the carbon dioxide pressure and hydrogen ion concentration of the arterial blood. The paradoxical alkaline and hypocapnic change in the blood in acute hypoxic hyperventilation is due to a specific sensitivity of the peripheral chemoreceptors to hypoxemia, causing an increased firing activity in the afferent chemosensory fibres. This increased firing leads to an increased activity of the respiratory center, which acts as a

relay. Its own, self-engendered activity (the centrogenic drive) decreases in acute oxygen deficiency, possibly due to the development of respiratory alkalosis. If respiratory compensation be intact, there is less reason now to postulate intracellular acidity in the center itself during acute hypoxic hyperventilation. However, evidence points to an acid change within the chemoreceptors as opposed to the hypocapnic and alkaline change in the blood. It is possible that the alkaline change influences to an important degree the outcome of chemoreceptor activity.

### 3. Some Particular Features of the Hyperventilation after Prolonged Exposure to Oxygen Deficiency.

The hyperventilation after prolonged exposure to oxygen deficiency seems to differ from that of acute hypoxia in some important respects. As has already been pointed out the alkaline change in the blood gradually swings back to a normal reaction, thereby causing the paradox of alkalosis and hyperventilation to disappear. This compensation is mainly brought about by a secondary decrease of the plasma bicarbonates. Although nothing is known about the chemoreflex activity after prolonged exposure to oxygen deficiency, some important findings as to the nature of the hyperventilation attach special interest to the rôle of the chemoreflexes. These findings will be reviewed below.

#### The Fixed Hyperventilation.

Contrary to its effect upon the acute hypoxic hyperventilation increased oxygen supply does not immediately decrease ventilation to its eupneic value when administered after prolonged exposure to oxygen deficiency. This was first observed by **BOYCOTT & HALDANE** (1908) in a series of experiments in a decompression chamber. They found that the alveolar carbon dioxide pressure, which had been lowered during a 24 hours' exposure to reduced barometric pressure, did not return at once to normal, but remained low for about two days. **OGIER WARD** (1908), who worked in conjunction with them, found the same thing, though in a much more marked and persistent degree, on returning to sea-level pressure after a stay on Monte Rosa. These early findings have recently been confirmed by **BECKER-FREYSENG, LOESCHKE, LUFT & OPITZ** (1942).

At present no generally accepted explanation to this "fixed hyperventilation" is available. PETERS & VAN SLYKE state (1931) that the hypoxic hyperventilation, "although initiated by the fall in atmospheric pressure, appears to reach its full development chiefly as a result of plasma bicarbonate diminution, which gradually reaches an extent that would occasion similar hyperventilation at sea-level". Such reasoning implies only an indirect importance of the oxygen lack for the hyperventilation after prolonged exposure, and not an immediate effect of want of oxygen as in the acute stage of hypoxic hyperventilation. This hyperventilation should accordingly be maintained essentially by the need for carbon dioxide removal, and should therefore be largely independent of sudden increase in the ambient oxygen pressure. The Y. HENDERSON theory on "respiration X" as the factor determining the hyperventilation in acute oxygen deficiency yields a similar explanation. BECKER-FREYSENG, LOESCHKE, LUFT & OPITZ, however, reject this reasoning, pointing out that a similar relative independence of increased oxygen pressure may prevail in subjects with no considerable decrease in their plasma bicarbonates.

It would be pertinent to question the rôle of the chemoreflexes in this insensitivity of the respiratory apparatus to increased oxygen. Remembering that the influence of acute oxygen deficiency upon the respiratory apparatus is entirely due to increased chemoreflex drive, one may suspect an adaptation of the chemoreceptors or the entire reflex mechanism after prolonged exposure to the hypoxic stimulus.

### The Effect of Additional Oxygen Deficiency.

It is well known since the investigations of LUTZ & SCHNEIDER (1919), that in human subjects the hyperventilation of acute hypoxia rarely surmounts the twofold eupneic value. This finding was confirmed by BENZINGER (1937) and by BECKER-FREYSENG, LUFT, LOESCHKE & OPITZ (1939, 1942). After prolonged exposure to moderate oxygen deficiency (at an altitude of 8,700—10,650 feet) it has, however, been observed that exposure to additional oxygen lack may increase the ventilation to about sevenfold its eupneic amount (BENZINGER, KAMINSKI & OPITZ, 1940, OPITZ 1940, BECKER-FREYSENG, LOESCHKE, LUFT & OPITZ 1942).

The mechanism of this increase in the excitability of the re-

spiratory apparatus to oxygen deficiency is not known. BENZINGER, KAMINSKI & OPITZ proposed an explanation according to which the mysterious substance "respiratory X" or "hyperpnein" (Y. HENDERSON) should be produced in increased amounts after prolonged exposure to oxygen deficiency. Significant is the finding of BECKER-FREYSENG, LUFT, LOESCHKE & OPITZ (1942), that the alkaline change in the blood caused by hyperventilation from additional oxygen deficiency is intensified, amounting to several tenths of a pH (an arterial pH of 7.7—7.8 was a common finding). The rôle of the chemoreflexes for this increased response of the respiratory apparatus to oxygen deficiency has not been inquired into. In view of the strong alkaline change in the blood, it is, however, highly desirable to investigate whether the center itself or the chemoreflexes are in main responsible for the increased hyperventilation. Contrary to the case in the last section (cf. "The fixed hyperventilation") one may be lead to suspect a very important rôle of the chemoreflex mechanism in the respiratory activity after prolonged exposure to oxygen deficiency.

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#### IV. The Problem.

The preceding review essentially constitutes an attempt to correlate present knowledge concerning the importance of the chemoreflex mechanism for the acute stage of hypoxic hyperventilation with earlier findings concerning the actual displacements in the acid-base balance of the blood. As a consequence of this correlation the problem of the present work has arisen, viz. the interaction of centrogenic and chemoreflex control of breathing in different stages of prolonged exposure to oxygen deficiency. A short recapitulation will reveal the motives for such an inquiry, apart from the fact that ignorance of the rôle of the chemoreflex mechanism during prolonged exposure to oxygen deficiency constitutes a reason per se.

1) During the course of prolonged exposure to a rapidly induced, constant and tolerated degree of oxygen deficiency in the inspired air at rest, a rapid, sometimes very marked, shift to increased blood alkalinity sets in. This shift is due to excessive blowing off of carbon dioxide, caused by the need for oxygen. The respiratory alkalosis is then gradually compensated by a decrease in the alkali in use as bicarbonates, so that the hydrogen ion concentration of the blood again attains a near to normal value. The time for full compensation varies individually from some days to some weeks. Considerable compensation should be accomplished within some hours. A schematic representation of the acid-base displacements in the blood during prolonged oxygen deficiency at rest is given in fig. 1.

2) Earlier theories on the chemical mechanism of hypoxic hyperventilation temporarily suffered in prestige as a result of the confusion, which followed the discovery of the chemoreflex mechanism. Granted certain modifications some of these theories can, however, be regarded as being as current today as they were before the discovery of the chemoreflexes. They all originated as attempts to explain the paradox of hyperventilation with hypocapnia and alkalosis in *acute* oxygen deficiency. Recent studies of the interaction of chemoreflex and centrogenic components in

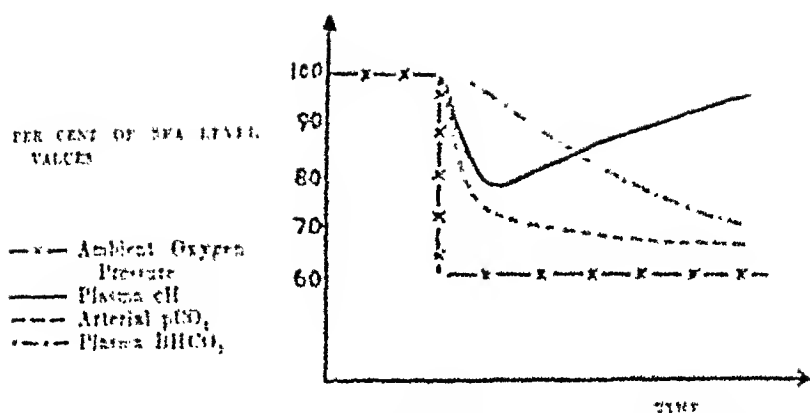


FIG. 1. Potential changes in the acid-base balance of the blood at rest during a rapidly induced, tolerated and constant degree of oxygen deficiency in the inspired air. The time factor is incompletely known for different degrees of oxygen deficiency and not important for the reasoning. The graphic representation is highly schematic.

acute hypoxic hyperventilation have revealed some unsupported relationships which seem to clarify some important details of the mechanism of acute hypoxic hyperventilation. Temporary cold-blocking of all afferent chemoreflex connections with the center during acute hypoxic hyperventilation has clearly demonstrated that the inherent, basal activity of the center is actually less than in eupnea, and may be zero in maximal hypoxic hyperventilation. The hyperventilation is entirely due to the chemoreflex drive. These findings have been confirmed by some of the experiments of the present work. It has been assumed, that the decrease of the self-engendered activity of the chemosensitive cells in the center itself in acute oxygen deficiency is due to the development of blood hypocapnia and alkalosis. However, experimental evidence is lacking on this point. Direct experimental approach to this question by means of continuous recordings of the arterial pH should reveal, whether the center actually reacts parallel to the change in the arterial  $p\text{CO}_2$  or  $\text{cH}$  during the development of respiratory alkalosis. Such recordings are included in the experimental procedure of the present work. The alkaline shift may amount to some tenths of a pH. One may ask what happens, when the hydrogen ion concentration of the blood moves down and up again during prolonged exposure to oxygen deficiency. Considering the well known response of the respiratory apparatus to increased arterial  $\text{cH}$ , the return towards neutral reaction, covering some tenths of a pH, would cause

a tremendous hyperventilation if the chemoreflex drive should remain as effective as in the stage of maximum alkalosis.

3) The hyperventilation in the acclimatized stage of oxygen deficiency has been stated to be of about the magnitude one would expect if a similar degree of hypocarbia were induced at sea-level. It has been shown that inhalation of oxygen at sea-level partial pressure after prolonged exposure to oxygen deficiency does not immediately restore pulmonary ventilation to its eupneic value, but only very gradually during a long period of new acclimatization to sea-level oxygen pressure. On the other hand it has been shown, that after prolonged exposure to oxygen deficiency, the respiratory response to additional oxygen lack is greatly enhanced, so that the hyperventilation is then several times greater than will ever occur from optimal oxygen deficiency, when acutely induced. These findings most conspicuously imply a change in the mechanism of hypoxic hyperventilation during the course of prolonged oxygen deficiency. On the one hand the respiratory activity is automatically speeded up, to a certain degree independent of increased oxygen supply (fixed hyperventilation); on the other its response to additional oxygen deficiency is greatly enhanced.

Therefore, since the chemoreflex mechanism seems to be the only intermediary in the stimulating influence of acute oxygen deficiency upon the respiratory activity, it is obvious that information on the chemoreflex drive after prolonged exposure to oxygen deficiency should be worth special interest.

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It may be said, that as far as acute hypoxemia is concerned, chemoreflex activity can be regarded as the most important single item in the body's defense. It is not unlikely, that many persons are alive today, because this activity was retained in conditions of a rapidly lowered oxygen pressure in the ambient atmosphere (aviators, mineworkers), in diseases with acutely impaired oxygen diffusion from the lungs into the blood (cases of pulmonary edema, pneumonia), in situations with an acutely reduced volume of oxygen that could be furnished to the tissues by the blood (cases of hemorrhage), and in the operating room, when narcotic drugs were given in large dosage. The rôle of the chemoreflexes in the hyperventilation only some hours after the onset of oxygen deficiency is, however, quite unknown. Considering

the motives, presented above, it seems therefore worth while to investigate, whether a change occurs in the balance of the centrogenic and chemoreflex components in the respiratory control during a prolonged exposure to a constant and tolerated degree of oxygen deficiency in the inspired air.

## V. Experimental Section.

### 1. Method.

The observations were made exclusively on dogs, anaesthetized with chloralose. (See below "Technique"). 27 dogs were used. The method of study mainly consisted in recording the relative effects upon the volume of breathing, the arterial pH and the arterial oxygen saturation of a temporary cold-blocking of the chemosensory impulses in the sinus nerves after short and prolonged exposures to a constant and tolerated degree of oxygen deficiency in the inspired air. An essential integrant of the experimental procedure was the arrangement for direct, simultaneous and continuous recordings of respiratory reactions and the pH and oxygen saturation of the circulating arterial blood (see below "Technique").

In the discussion accounts will also be given of some auxiliary experiments of the present work, the results of which have contributed to the interpretation of the main results.

In the blocking experiments the vagus nerves were sectioned to prevent permanently those signals arising in the aortic pressoreceptors and chemoreceptors from reaching the respiratory centers. The blocking of the sinus nerves therefore eliminated all known remaining afferent chemoceptive impulses.

Experiments with sinus nerves temporarily blocked during oxygen deficiency have recently been made by GESELL, LAPIDES & LEVIN (1940). These experiments applied to the acute stage of hypoxia only, and did not involve measurements or recordings of arterial pH and oxygen saturation.

Blocking the impulses in the sinus nerves (vagi cut) can theoretically have the following effects upon breathing: 1) withdrawal of inhibition from the carotid pressoreceptors 2) withdrawal of carotid chemoreflex drive, and 3) a combination of 1) and 2). It is therefore necessary to eliminate permanently the pressor impulses in the sinus nerves, before the amount of chemoreflex drive can be judged from the respiratory reactions, which occur during and after the blocking. Otherwise, should the inhibitory

action of the pressoreceptors be greater than the excitatory action of the chemoreceptors, an increased volume of breathing would be expected to occur during sinus nerve block (fig. 7 A). In the experiments of GESELL et al. the signals from the pressoreceptors were permanently eliminated by collapsing the sinuses. The sinuses were relieved of their normal distension by tying ligatures 1) around the common carotids just below the sinuses, 2) around the internal carotids just distal to the sinuses, and 3) around the external carotids as close to the sinuses as possible and caudal to the origins of the occipital arteries (after GOLLWITZER-MEIER, 1934). The common carotids were then punctured peripheral to ligatures 1). From the findings of WINDER (1933) and WINDER, BERNTHAL & WEEKS (1938) it was assumed that the remaining anastomoses would act to preserve a uniform flow of blood through the carotid bodies in spite of the occlusion of the common carotids. The author agrees that the blood flow may be near to normal if the systemic arterial pressure is high, as it tends to be after the elimination of all afferent pressosensory impulses. But in the case of lowered arterial pressure with stagnation of the blood flow in the anastomoses, the possibility of respiratory effects from chemoreceptor excitation as a result of local accumulation of acid metabolites is obvious (EULER & LILJESTRAND 1937, EULER, LILJESTRAND & ZOTTERMAN 1939, EULER & LILJESTRAND 1940, BJURSTEDT & HESSER 1942, and BJURSTEDT & EULER 1942).

The author has therefore preferred to keep the blood supply to the carotid bodies intact. The carotid pressor impulses were eliminated by carefully sectioning with a fine pair of scissors the pressosensory fibers originating in the sinuses on their way to join the fibers from the carotid body (see below "Technique"). After the vagus nerves had been sectioned the respiratory center was thus free from all known influence of peripheral pressoreceptor excitation, and the cold-blocking of the sinus nerves thus eliminated all remaining chemoreflex drive selectively.

It has been shown recently by BERNHARD & GRANIT (1946), that local cooling of mammalian nerves may temporarily set up impulses from the cooled part of the nerve. The possibility of an actual excitation of the sinus nerve during cooling has therefore been taken into special consideration in the present work. It is obvious that, even if a part of the sinus nerve be so rapidly cooled that there is no reason to expect respiratory effects

from a transient spontaneous discharge at that part of the nerve, there may theoretically be some adjacent part of the nerve central to the place of cold-blocking, that is only slowly cooled. If it be so, one would actually expect effects from excitation of both pressosensory and chemosensory fibers central to the cooled part of the nerve. The outcome of such excitation has, however, been none or insignificant in the present experimental procedure. It has been observed that cold-blocking the sinus nerves with the pressosensory fibres intact and the vagi cut always produced a great rise in the arterial pressure (fig. 7 A). One would expect a lowering, if the pressosensory fibres were excited to a significant degree. Regarding the possibility of excitatory effects from the chemosensory fibers it was observed that, after disconnection of the vagi and carotid pressoreceptors, blocking of the sinus nerves produced either a decrease in breathing or no respiratory reaction at all. One would expect increased breathing during the block if significant effects from excitation of chemosensory fibers occurred.

It is therefore concluded that the blocking procedure employed in the present work actually yielded clean-cut effects from a functional disconnection of all remaining chemoreflex drive.

## 2. Technique.

### Preparatory Procedure.

As has already been mentioned dogs were used throughout as experimental animals. The weight varied from 10 to 30 kg. The animals were anaesthetized with chloralose intravenously in doses of about 10 ml per kg of a 1 per cent solution. In some cases it was found necessary to add some ml of a 20 per cent solution of urethane in order to stop excessive shivering. Since the duration of the experiments was usually between 6 and 10 hours further doses of chloralose had to be given at intervals of some hours in order to keep the anaesthesia at a uniform depth. The anaesthesia was always slight and controlled so that reflex motor reactions were just suppressed.

The carotid regions were exposed without reflecting the larynx. By blunt dissection these regions were carefully freed from other nervous connections than the sinus nerves. All severed nerves were carefully ligated in order to prevent spontaneous discharge, which would otherwise affect the respiratory activity reflexly when the tissue was necessarily stretched during manipulations in this region. Small open vessels were scrupulously ligated in order to avoid hemorrhage, which would otherwise be considerable on account of the use of large doses of heparin. The vagi were cut between ligatures. The next step

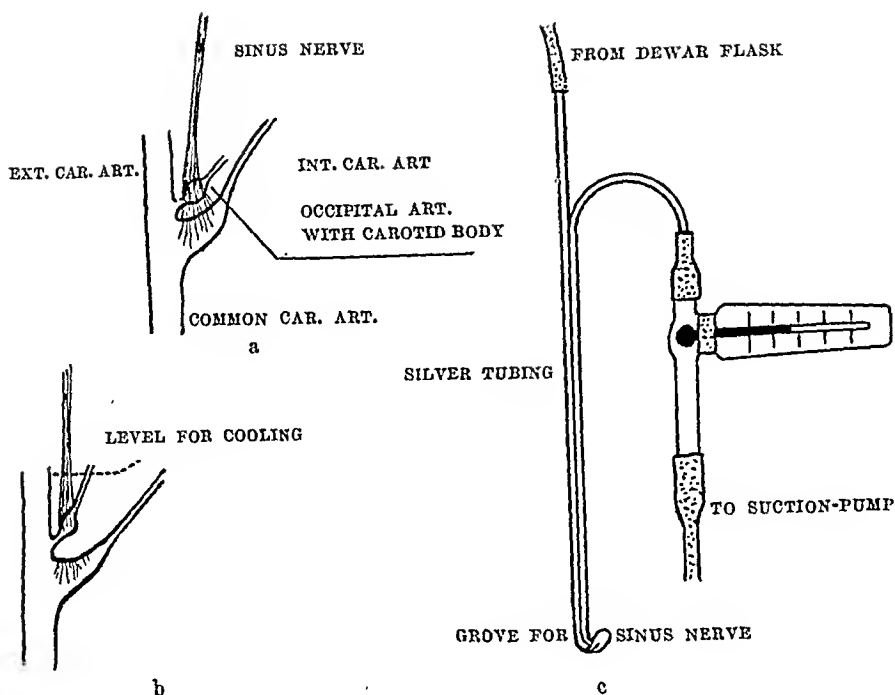


Fig. 2.

- a. Medial aspect of bifurcation of right common carotid.
- b. Bifurcation region after severing of pressosensory fibres.
- c. Cold-blocking device.

was the severing of the pressosensory fibers from the sinus regions (fig. 2). This was done with a fine pair of scissors. The sinus regions were first denuded so that the pressosensory fibers could directly be seen as fine whitish bundles. Then the fibers were carefully sectioned bundle by bundle on their way to the carotid body. Extreme care was taken not to injure the carotid bodies. None the less this was sometimes done and the animal was then of no value for the purpose, if it did not show any respiratory response to oxygen lack. It was gradually found that even if the pressosensory fibers had not completely been disconnected, the remaining fibers soon ceased their tonic influence spontaneously, apparently because they had been injured. The gradual disconnection was followed during the denervation procedure by observing the gradual increase of arterial blood pressure. The completeness of the denervation was proved by the disappearance of the pressor effect, that normally results from cooling the sinus nerves and from occlusion of the common carotids. When the pressosensory fibers had been severed the sinus nerves were freed from superfluous tissue. A cord of tissue the thickness of a match was, however, left surrounding the nerve in order to avoid injury. It was essential that no fibers originating in the carotid body could escape from this



cord of tissue, passing along the carotid vessels to other central connections. Therefore, the walls of the carotid vessels were carefully denuded.

### Cold-blocking Technique.

The chemosensory impulses in the sinus nerves were effectively blocked by cooling the cords of tissue containing the sinus nerves by special devices (fig. 2 c). Rapidly circulating alcohol of 0 to  $-1^{\circ}\text{C}$  was used for the blocking. Deblocking was caused by shifting to alcohol of  $35-38^{\circ}\text{C}$ . Between blockings the devices were usually removed. Temperature changes between  $37^{\circ}\text{C}$  and  $-1^{\circ}\text{C}$  required some seconds. The moment of blocking and deblocking was signalled when the temperature reached  $0^{\circ}\text{C}$  and  $20^{\circ}\text{C}$  respectively. Incidentally it was found that the nerves were permanently damaged if a blocking temperature of less than  $-3^{\circ}\text{C}$  was used. The blocking devices were applied about a half to one centimetre central to the carotid body.

### Recording and Measuring Apparatus.

For the present purpose a special measuring and recording system was constructed. It consisted of an optical system including a camera and of devices for direct, continuous and simultaneous recordings on photographic paper of 1) volume of breathing, 2) arterial pH, 3) arterial oxygen saturation, and 4) arterial blood pressure. The electrical recordings of 1), 2) and 3) represent new designs. A schematic representation of the recording arrangements is given in fig. 3.

#### a) *Volume of Breathing.*

Two different methods were alternatively used for the recording of the volume of breathing (fig. 4). One method consisted in having the dog breathe in a KROGH's respirometer with soda-lime for the absorption of carbon dioxide. The oxygen content in the lungs of the dog and the respirometer was kept constant by supplying the closed system with just the quantity of oxygen required to prevent the indicator of the respirometer to sink from breath to breath (oxygen added as used). The oxygen content was repeatedly controlled by gas analysis. The movements of the respirometer roof were transmitted to a U-bent glass capillary on which was mounted a thin platinum filament. The legs of the capillary with the platinum filament dipped into two glass-tubes filled with mercury. When the capillary moved up and down with the roof of the respirometer the electrical resistance between the mercury portions changed linearly. The resistance changes were directly recorded by means of a Wheatstone bridge arrangement and a torsion band oscillograph. Thus the volume of breathing could in a simple electrical way be quantitatively recorded on photographic paper. It was observed that a part of the hot platinum filament easily

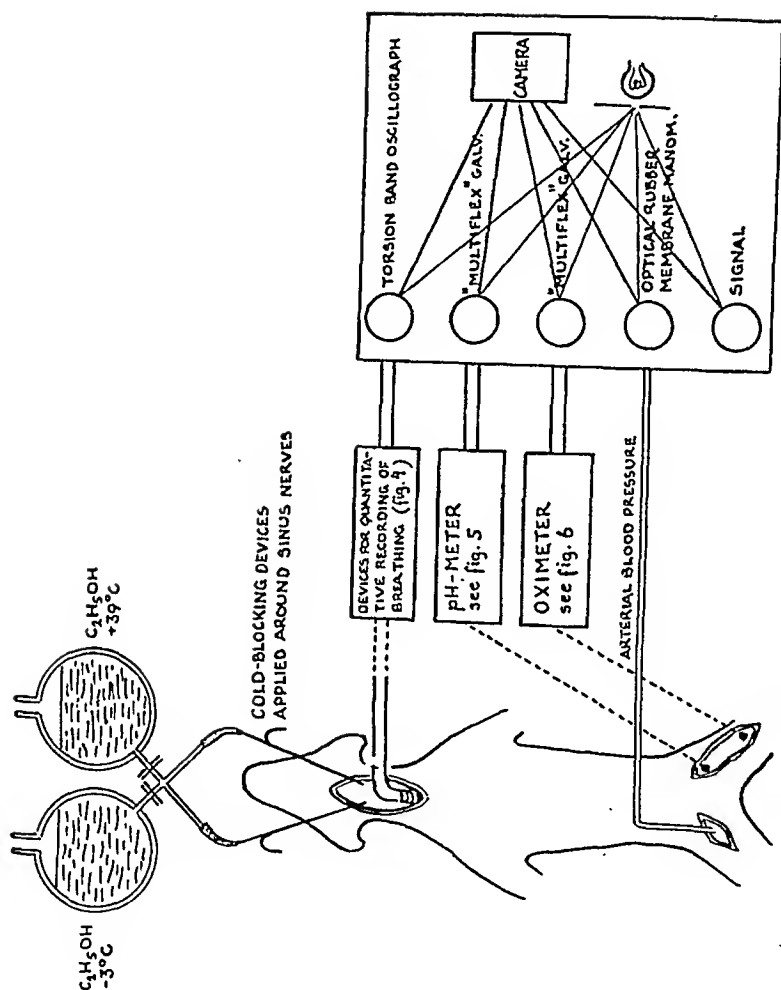


Fig. 3. General schema of experimental arrangement.

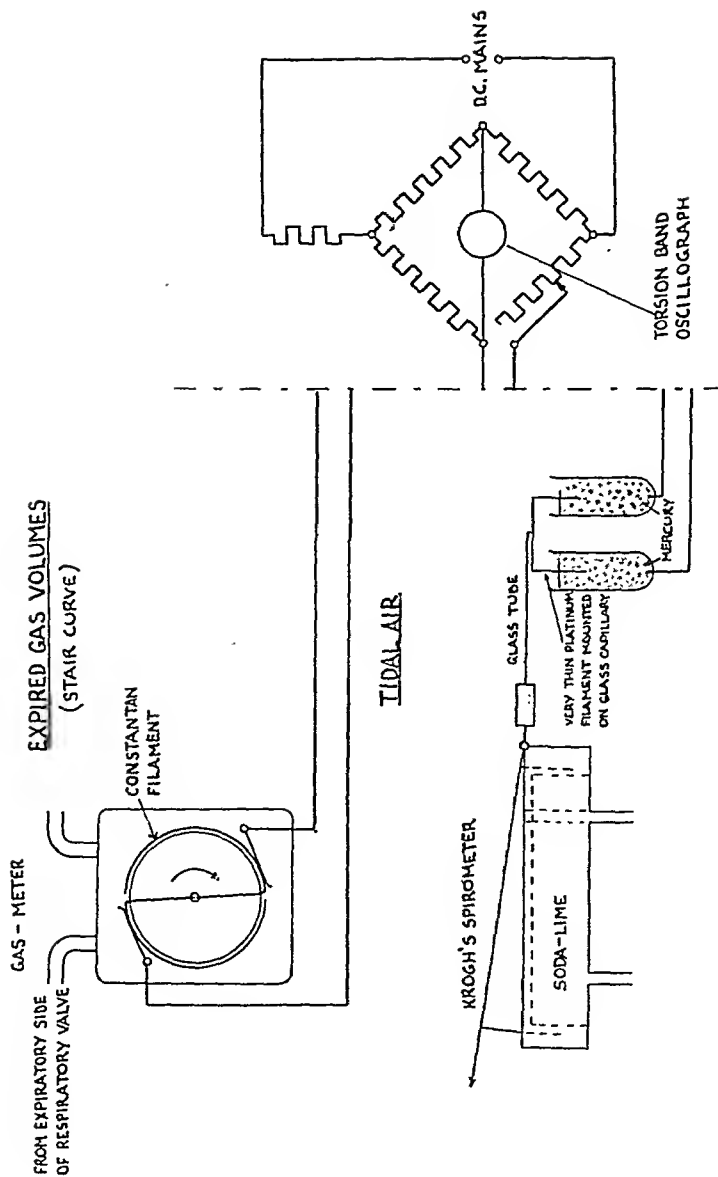


Fig. 4. Methods of recording quantitatively the volume of breathing.

became covered by a thin layer of mercury oxide, causing instability in the contact between the mercury and the platinum. This part of the filament was found to correspond to its excursions in relation to the mercury surface. It was also found, however, that a layer of undiluted sulphuric acid covering the surface of mercury dissolved the coating of the filament most efficiently, so that the contact remained self-cleansing.

The other method of recording the volume of breathing was a simplification of a method previously described by KRAMER & GAUER (1941). A gas meter was connected to the expiratory side of the respiratory valve. A light wheel was fastened to the axle of the meter, and on the periphery of the wheel there was a constantan filament, bent as an S. At opposite sides of the wheel silver plate contacts were applied. When the wheel moved with each expiration the electrical resistance between the contacts changed linearly, and the volume of breathing could thus be electrically recorded in the same manner as above, although only the expiratory strokes were recorded (stair curve).

#### b) Arterial pH.

Earlier reports on direct and continuous recordings of the pH of the circulating blood are few. It seems that no investigations have been published, which involve such recordings for more than short periods. The main difficulty is to ensure constancy of the electronic transmission of the hydrogen ion concentration potentials, i. e. to prevent a spontaneous drift of the zero point of the amplifier between the measuring and reference electrodes and the oscillograph. After laborious experiments the following method was finally adopted. Two AEG electrometer tetrodes T114 were used in a bridge connection. They were specially selected to be as identical as possible. The AEG T114 electrometer tetrode has a very small grid-current ( $10^{-14}$  amps.), and is therefore most suitable for measuring of pH potentials. The cathode filaments were connected in parallel and heated from the same source of supply as delivered the anode and space charge-grid currents. The negative control grid bias was of the automatic cathode type for both tubes, and a resistor in series with the cathode filaments acted as common cathode resistor. Thus all the electrodes of the tetrodes could be supplied from one single voltage fall (A—B), provided by an A. C. power supply, including a two-section filter with a choke input. Further voltage stabilization was effected by a gaseous regulator tube, Philips 13201. In case of extreme fluctuations in the line voltage of the town, the A—B voltage was provided by a storage battery of 16 volts instead. The current consumption of the pH-meter was 0.166 amperes. By careful adjustment of the space charge-grid voltage of one of the electrometer tetrodes the drift of the zero point of the pH-meter could be reduced to some millivolt during the usual continuous use of at least 6 hours. See wiring diagram, fig. 5.

The glass electrode assembly was built according to fig. 5. This design was found to be handy and stable. The inlet and outlet tubes

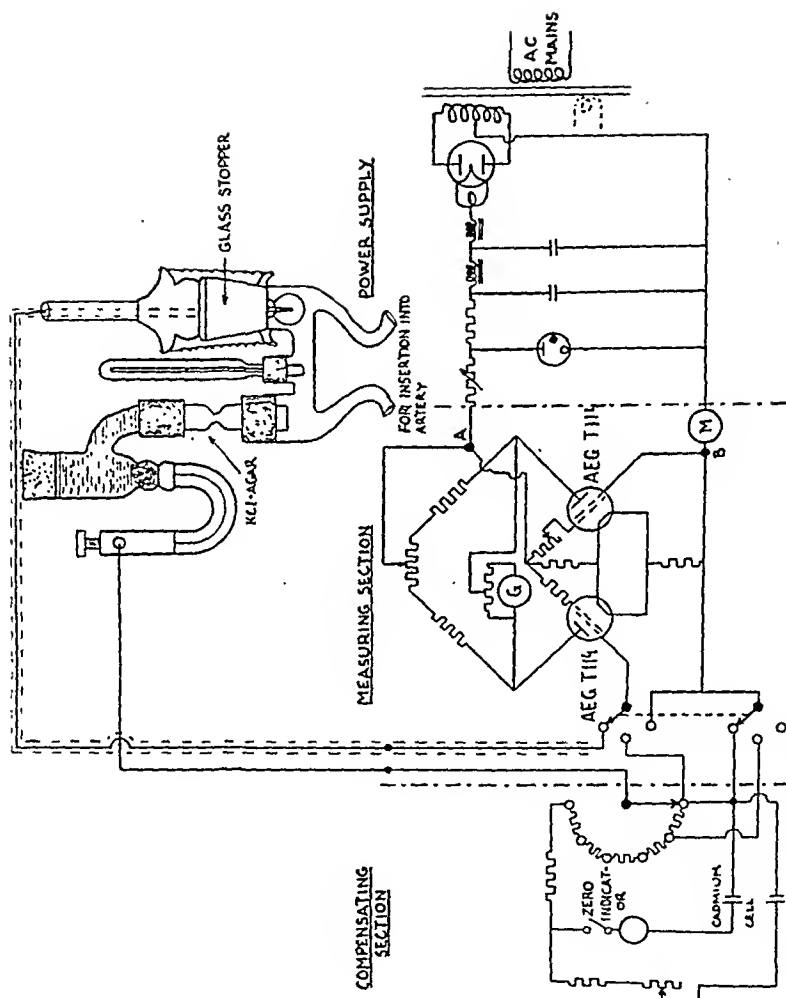


Fig. 5. Schematic representation of A. C.-powered device for measuring and recording continuously the pH of the circulating arterial blood.

were connected to the chamber so that the blood stream automatically prevented sedimentation of blood corpuscles. In order to prevent clotting, the inside of the glass electrode chamber was coated with a thin layer of wax, dissolved in ether. Coagulation was further prevented by 1 ml of a 5 per cent solution of heparin per 10 kg body weight intravenously. The glass electrode chamber was inserted into the left femoral artery.

The changes in the pH of the arterial blood was recorded by means of a Multiflex galvanometer, type MG 3, which was connected between the anodes of the electrometer tetropdes. A deflection of 12 mm on the photographic paper corresponded to a pH change of 0.1. The pH-meter was calibrated by switching the glass and reference electrodes to a commercial pH-meter (Radiometer, Copenhagen), on which momentary pH values could be read on a dial. The temperature of the blood in the glass electrode chamber was allowed for. For this purpose a small thermometer was fitted into the glass electrode chamber.

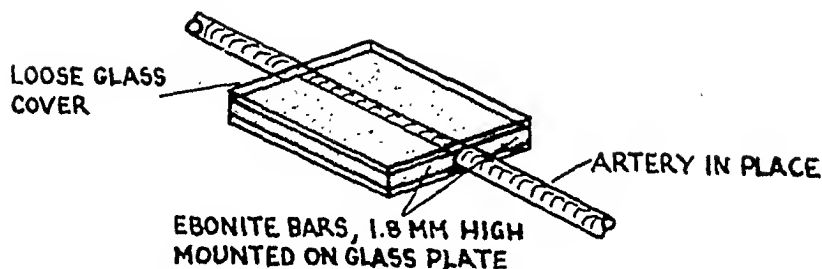
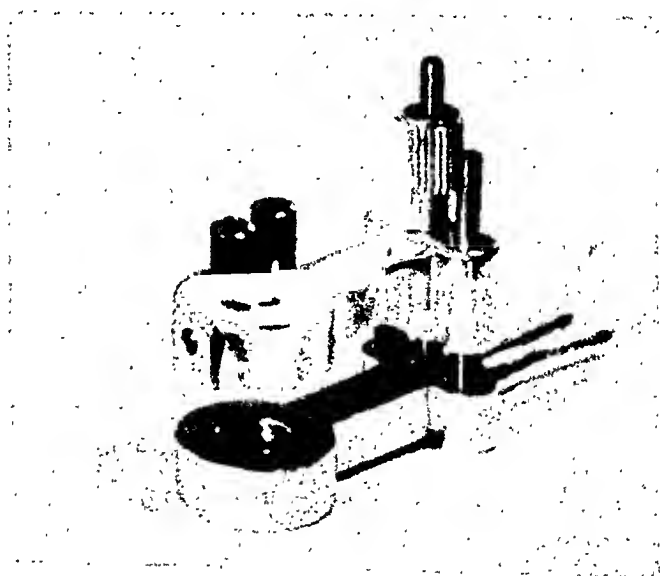


Fig. 6. Oximeter unit and glass holder with artery.

### c) *Arterial Oxygen Saturation.*

For measuring and continuously recording the oxygen saturation of the arterial blood the photo-electric oximeter was used. The principles of the oximeter have been described by KRAMER (1935). The form designed for the present purpose is shown in fig. 6. It consists of a small U-frame, made of aluminium, bearing on one leg a 4-volt miniature lamp bulb and carrying on its other leg a small Bakelite box containing a barrier-layer red-sensitive photo-cell (Lange, type SK). The U-frame slips over a glass holder for the artery, the glass holder being clamped between the legs of the frame by a screw arrangement. The glass holder was designed so as to prevent light to pass by the artery. The supply to the lamp was provided by a high capacity storage battery (60 ampere-hours). The lamp was switched on *in situ* some hour before use to ensure constant light source and constant temperature of the photo-cell. Usually the left femoral artery was used. The photo-cell was directly connected to a Multiflex galvanometer, type MG 3. The sensitivity of the oximeter could be varied by altering the current supply to the lamp. The oximeter was calibrated by means of van Slyke analyses of blood samples, which were obtained from a branch of the femoral artery. Cooling of the oximeter, as done in KRAMER's version, was found to be unnecessary, since the present design ensured sufficient constancy of the temperature of the lamp and photo-cell by the cooling action of the circulating blood and the ambient air.

### d) *Arterial Blood Pressure.*

The arterial pressure was recorded by means of an ordinary optical rubber membrane manometer. The manometer was connected to the right femoral or to the left brachial artery.

## 3. Introductory Remarks.

Before the main results are described and discussed (p. 60) some preliminary questions have to be considered. Some of these questions do not refer directly to the main subject of the present work, but had to be considered in order to ensure uniformity of the experimental conditions. Other preliminary questions to be examined have certain bearings upon the interpretation of the main results.

### *Influence of the Anaesthetics.*

The depressing action of chloralose and urethane upon the center is well known (see the basic work of MARSHALL & ROSENFELD 1936, for review see BERNTHAL 1944). SCHMIDT (1940)

states that chloralose contributes to the unbalance between centrogenic and chemoreflex drives not only by the relative increase of the chemoreflex drive resulting from a depression of the central response to carbon dioxide, but furthermore by actually exaggerating the chemoreflex response to hypoxemia. In profoundly anesthetized animals the maintainance of respiration should mainly, if not entirely, be effected by the chemoreflex drive, elicited by the «normal» hypoxemia, and inhalation of oxygen would accordingly cause apnea and eventually respiratory failure (MARSHALL & ROSENFELD, 1936, BENZINGER, OPITZ & SCHOEDEL, 1939). The cause of this insufficiency is obscure, for even if pure oxygen is inhaled, and a decrease of the respiratory activity is thus induced, a point of arterial oxygen unsaturation will soon be reached, where excitation of the chemoreceptors again occurs. Certainly the continuing respiratory insufficiency can not be explained by assuming a decrease of chemoreceptor excitation. As the result of increased oxygen supply some factor must apparently have been added to the mechanism, by which the outcome of chemoreceptor excitation is diminished. We will return to this subject in conjunction with the discussion of the results of the present work. Anyhow, the disturbing influence of general anaesthesia upon the balance of components in the respiratory control is an established fact.

It is obvious that a change in the balance between centrogenic and chemoreflex components may occur as the results of a mere change in the depth of the anaesthesia, other things remaining constant. For instance, the depression of the centre should be decreased as the result of a decrease in the depth of the anaesthesia, such as will occur spontaneously during the course of sufficiently prolonged experiment.

This undisputed fact has important bearings upon the present work. It is not likely, however, that a change in the balance between centrogenic and chemoreflex components will occur as a mere result of a spontaneously changing depth of anaesthesia, if the observations be made within the course of an hour or two, and the anaesthetics used be chloralose and urethane. Such a spontaneous change in the balance between the centrogenic and chemoreflex drives was never observed during such short periods, if the animal was not breathing gas mixtures with a changing oxygen or carbon dioxide content. But if the observations be extended to later stages in the anaesthesia the possibility of a



spontaneous change in the balance of centrogenic and chemoreflex drives must be taken into consideration.

Hence, in those experiments of the present work where the influence of a constant degree of prolonged (4—8 hours) ambient oxygen deficiency was to be observed, it was essential that the depth of the anaesthesia was not allowed to decrease during the period of observation. As has been mentioned, this was done by adding more chloralose at intervals of one hour or more and in amounts, that were just effective in suppressing reflex motor reactions. Usually such a procedure would ensure a steady ventilation, be it eupneic or increased by oxygen deficiency. In some instances it was found that a spontaneous increase in the respiratory activity would be the first signal of a decreasing depth of the anaesthesia. It is not unlikely that this increase was the result of excitation of non-specific sensory nerves in injured regions. In these instances the respiratory response to varying depth of anaesthesia would be considerable. Such animals, or, rather, such preparations, were accordingly not suitable for prolonged observations, even if the unavoidable variable of a changing depth of anaesthesia could be reduced. For prolonged observations, therefore, only such animals were selected, which showed a steady ventilation, relatively insensitive to the degree of anaesthesia induced.

The question of the influence of the anaesthetics upon the absolute amounts of centrogenic and chemoreflex components under varying conditions is, of course, of minor importance in the present work, since the discussion essentially refers to the changes in the mutual relationship.

### **The Arterial Oxygen Saturation.**

It was observed that in prolonged experiments abundant bleeding would gradually cause a dilution of the blood, that reduced the accuracy of the oximeter readings. For that reason hemorrhage had to be prevented by painstaking ligation of bleeding vessels in injured regions of the animal. This was especially necessary since the animals were given heparin in large dosage. For the same reason abundant administration of fluid intravenously was also avoided. A total administration of some hundred ml of an isotonic solution of glucose during the course of a prolonged experiment did not, however, cause any distortion of the oximeter

readings from dilution of the blood, Hence, the oximeter usually needed calibration only once during an experiment.

### **The Arterial Blood Pressure.**

Several interesting effects upon the arterial blood pressure were observed as the result of the blocking experiments. Usually, the onset of the blocking caused an immediate decrease of the arterial pressure. During the period of blocking the arterial pressure would again gradually rise a little. On deblocking, the arterial pressure returned to its former level, if this had not already happened. The initial decrease in the arterial pressure obviously results from the disconnection of a tonic drive from the chemoreceptors in the maintenance of the arterial pressure (cf. above »Method»). The effects upon the arterial pressure obtained in the experiments of the present work will be described and discussed in a subsequent paper. At present only such effects are described that are of importance for the interpretation of the respiratory reactions.

### **The Chemoreflex and Centrogenic Drives in Eupneic Breathing under General Anaesthesia.**

As has been mentioned above (p. 28) evidence points to the chemoreceptors acting as pace-makers for the respiratory apparatus even during eupneic breathing. In the present work the results of the blocking experiments clearly confirm earlier findings on this point. Even in cases of high arterial oxygen saturation the blocking of the impulses originating in the carotid bodies caused an immediate decrease of the respiratory activity (fig. 9 A). As the results of this decrease in the volume of breathing the arterial oxygen saturation decreased and the cH of the blood increased somewhat. After the deblocking the volume of breathing immediately increased again, usually to a magnitude slightly greater than before the period of blocking. This increase above the eupneic amount was obviously due to the effect upon the chemoreceptors and centre of the increased arterial cH or  $p\text{CO}_2$ , since the volume of breathing gradually returned to the former eupneic magnitude parallel with the arterial cH. Hence, the pace-making influence of the chemoreflexes in the maintenance of the arterial oxygen saturation is obvious.

### **Tolerated Degree of Ambient Oxygen Deficiency under Anaesthesia.**

For the present purpose it was necessary to determine the degree of ambient oxygen deficiency, that would — under slight chloralose anaesthesia — bring about a distinct increase in the pulmonary ventilation and at the same time be tolerated, so that respiratory depression would not gradually develop. It was found that a gas mixture of about 8 per cent oxygen in nitrogen usually produced an increase of the ventilation in dogs, that were chemoreceptively intact, to about the twofold eupneic amount, and that this degree of ambient oxygen deficiency was usually tolerated for periods as long as 10 hours, which was the longest exposure needed for the present purpose. In some animals, which had been deprived of their vagal pressosensory and chemosensory connections and of their carotid pressosensory connections, the tolerance was found to be diminished. This fact may have been due to the vagotomy and/or to the damage eventually done to the carotid chemoreceptors during the sectioning of the carotid pressosensory fibres. Lower oxygen percentages than 10 in the inspired air were often not tolerated for sufficiently long periods by these dogs. A quite sufficient degree of hyperventilation was, however, usually produced by 10—12 per cent oxygen in these cases.

The effect of bilateral vagotomy during breathing of room air is shown in fig. 7 B. It is obvious that the sudden shock causes a temporary, rather considerable, respiratory alkalosis, and that the arterial oxygen saturation, after a temporary lowering, returns to its former eupneic level. The vagal connections seem to have no significant rôle for the maintenance of the eupneic arterial oxygen saturation. From the above it is, however, probable that their importance is increased during exposure to low oxygen.

### **The pH of the Arterial Blood before, during and after Hypoxic Hyperventilation.**

The maximum alkalotic deviation in the arterial pH, resulting from a rapidly induced, constant and tolerated degree of oxygen deficiency in the inspired air was found to be closely dependent on the degree of oxygen deficiency. In dogs with their pressoreceptive and chemoreceptive connections intact an oxygen content in the inspired oxygen—nitrogen mixture of 8—10 per cent

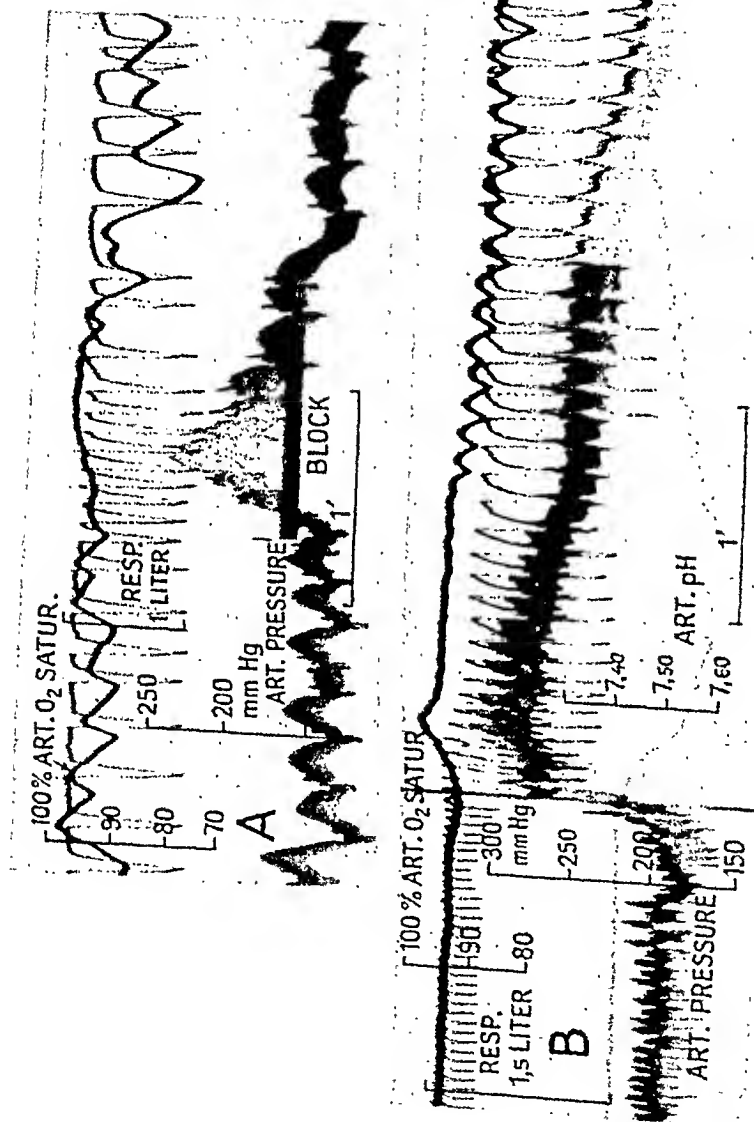


Fig. 7. A. Effects of blocking the chemoreceptor and pressoreceptor signals on pulmonary ventilation and arterial pressure. Breathing of room air, vagi cut. Great increase in pulmonary ventilation and arterial pressure from withdrawal of pressoreceptor inhibition. Compare with other figures, where the blockings were made with pressoreceptor fibres disconnected. B. Effects of sectioning both vagal nerves (at double line) in the laryngeal region during breathing of room air. Note return of arterial pH and O<sub>2</sub> saturation after temporary disturbance.

usually caused a maximum deviation of pH  $+0.1$  to  $+0.25$ . Dogs with sectioned vagi and carotid pressosensory fibres usually developed an alkalotic deviation amounting to about a tenth of a pH unit on breathing oxygen at about 11 per cent, this decreased deviation of course depending upon the minor increase in the respiratory activity. (It was mentioned above that the tolerance for decreased oxygen in the inspired air was less in the partially denervated dogs than in the intact ones.)

The rapidity with which the maximum respiratory alkalosis developed, when constant ambient oxygen deficiency was rapidly induced, varied from animal to animal, but seemed to be rather independent of the degree of oxygen deficiency and of the readiness with which the animals responded to oxygen deficiency. Usually the maximum deviation was reached within a quarter of an hour to one hour.

If the exposure to a constant degree of oxygen deficiency in the inspired air was suddenly discontinued during the development of the alkalotic change or at a point when the alkaline change was at its maximum, the arterial pH would fairly rapidly return to its prehypoxic level. The return was usually complete within 5 to 15 minutes. If the exposure was instead prolonged the maximum deviation would remain constant for a period of time that varied individually, but seemed to be rather independent of the ability of the animal to respond to oxygen deficiency with hyperventilation. The maximum respiratory alkalosis, however, was usually retained for several hours, before compensation slowly developed. A full spontaneous return of the arterial pH to its prehypoxic value during continuous exposure to a constant degree of oxygen deficiency in the inspired air was always, sooner or later, observed. The following can be said on the nature of this compensation. If the hyperventilation was retained until the arterial pH became spontaneously normal again, the alkalosis must have been compensated by a decrease in the buffering capacity of the arterial blood. It is true that in most cases of the present work, there was hyperventilation when the prehypoxic arterial pH was again gradually attained. But it is also a common experience that in prolonged experiments on animals under general anaesthesia the pulmonary ventilation is likely to decrease in the last stages, when the condition of the animal becomes poor. In such cases the factor "respiratory acidosis" partially determines the actual acid-base condition in the arterial blood. It is clear, therefore, that in late

stages of hypoxic hyperventilation under anesthesia, such as was induced in the present experiments, the prehypoxic arterial pH can theoretically be assumed to return, not only as the result of a secondary decrease of the alkali in use in the blood (cf. above p. 15) but also as a result of the additional factor of respiratory acidosis. But as long as the hypoxic hyperventilation is continuously going on, the factor of respiratory acidosis need not be taken into consideration. Those breathing reactions which have been observed in the present work concerning the late stage of hypoxic hyperventilation were always provoked at a point when the respiratory alkalosis was nearly compensated without any sign of respiratory depression. The observed cases therefore represented the compensation of respiratory alkalosis by a decrease of the buffering capacity of the blood and not the compensation brought about by decreased respiratory activity. The question as to whether the decrease of the buffering capacity depended to a certain degree upon increased liberation of acid metabolites ("metabolic acidosis") besides the non-acidotic compensation (cf. above p. 15) has not been answered by the investigations in the present work. It is, however, not unlikely that "metabolic acidosis" was also acting.

Usually 4 to 8 hours of hypoxic hyperventilation (the inspired gas mixture contained 8—12 per cent oxygen in nitrogen) had to pass before a stage was reached, when the abovementioned requirements were fulfilled. The pH of the arterial blood had then nearly, but not completely, returned to its prehypoxic level, and no sign of respiratory depression prevailed.

If room air was substituted for the oxygen-poor gas mixture when this late stage was reached, the pH of the blood showed a definite shift to acidosis. This acidotic shift was, however, only transient, and the pH essentially again returned to the prehypoxic value within varying periods of time.

These findings referring to the pH of the arterial blood before, during and after prolonged exposures to oxygen deficiency in the inspired air essentially coincide with earlier experience concerning unanaesthetized subjects (mountaineers: alkalosis on ascent — acidosis on descent, see HENDERSON, 1938) and anaesthetized animals (cf. p. 15).

A schematic representation of the essential changes in the arterial pH of the anaesthetized dog during and after short and prolonged exposures to a constant and tolerated degree of oxygen

deficiency in the inspired air is given in fig. 8. In this diagram four points are indicated by the letters A, B, C and D. These points mark the main moments, at which the effects of blocking the chemoreflex drive (and other respiratory reactions) have been studied in the present work (see below "Results and discussion").

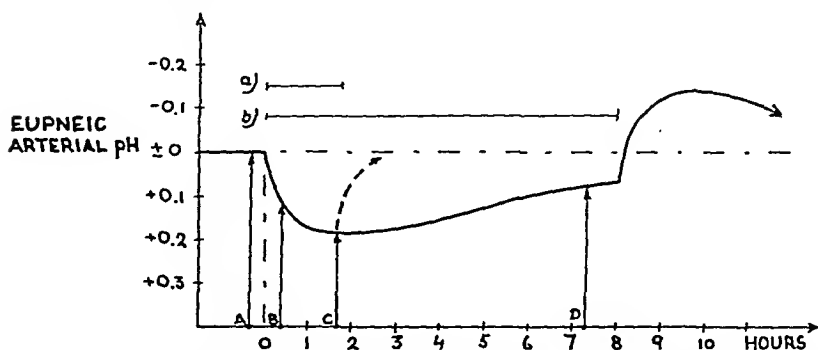


Fig. 8. Important levels of arterial pH during the course of a prolonged exposure to constant ambient oxygen deficiency, at which the interaction of centrogenic and chemoreflex components has been studied in the present work: eupnea — point A, developing respiratory alkalosis — point B, maximum alkalosis — point C and the point of nearly complete compensation — D.

a) = acute, b) = prolonged exposure. (From a dog, that was subjected to 8,2 %  $O_2$  in  $N_2$ .)

#### 4. Results and Discussion.

**Acute Oxygen Deficiency: Interaction of Centrogenic and Chemoreflex Components in Hypoxic Hyperventilation during the Stage of Developing Respiratory Alkalosis.**

##### *The Center.*

It is conceivable that in those conditions where the respiratory activity is partly maintained by chemoreceptor excitation the chemical control of breathing divides up in two components, viz. the centrogenic and the reflexogenic chemical drives. If at any moment the impulses in the peripheral chemosensory fibers be completely blocked the volume of breathing must immediately decrease and the new volume of breathing is controlled only by the self-engendered activity of the chemosensitive cells of the center. This centrogenic chemical control of breathing will last till the chemoreflex chemical control is re-established by a deblocking of the peripheral chemosensory impulses.

The importance of the chemoreflexes for the acute hypoxic hyperventilation is well established. On the rôle of the centre during acute hypoxic hyperpnea opinions, however, still differ

greatly. Mainly as a consequence of the missing response of the chemoreceptively denervated centre to hypoxemia (p. 31) an idea has developed that hypoxemia produces a condition of decreased vitality, "paralysis", of the vegetative functions of the centre, even when the chemoreflexes are intact. Another conception, derived from the second reaction theory of WINTERSTEIN (p. 19), maintains that the centre has a share in the hypoxic hyperpnea, and that this share is evoked by increased intracellular acidity from local anaerobic production of lactic acid.

The blocking experiments of the present work appear to have revealed the rôle of the centre for the acute hypoxic hyperventilation. It was found that even under eupneic breathing the blocking of the peripheral chemosensory impulses caused a sudden decrease in the volume of breathing. Fig. 9 A points to the importance of the chemoreflexes for the maintenance of the normal oxygen saturation under general anaesthesia at sea-level (cf. p. 28). When the ambient oxygen pressure was reduced and hyperventilation was thus set up, blocking of the chemoreflexes revealed that the centrogenic support was now definitely less than during eupnea (fig. 9 B). If the chemoreflexes were blocked during acute hypoxic hyperventilation at a stage when the alkaline change in the blood had become maximal, the volume of breathing always immediately and suddenly decreased, sometimes to apnea. This must mean that the centre has no share in the acute hypoxic hyperventilation, for otherwise there would have been supernormal centrogenic breathing from the moment when the block took effect.

The question now arises, why the centrogenic breathing is less in acute hypoxic hyperventilation than in eupnea. An answer to this question is given by the following observations. First it was found that the effect of oxygen deficiency *after* the centre had been chemoreceptively denervated is fundamentally different from the effect of sudden denervation (by blocking the chemoreflexes) *during* acute hypoxic hyperventilation. It is well known that in the first case the volume of breathing only gradually decreases, conceivably due to the devitalizing effect of hypoxemia upon the centre. Temporary "denervation" during acute hypoxic hyperventilation, however, always decreased the volume of breathing suddenly. Remembering now that this new volume of breathing, "centrogenic breathing", was always less than the centrogenic breathing in eupnea (see above), one may inquire if this decrease



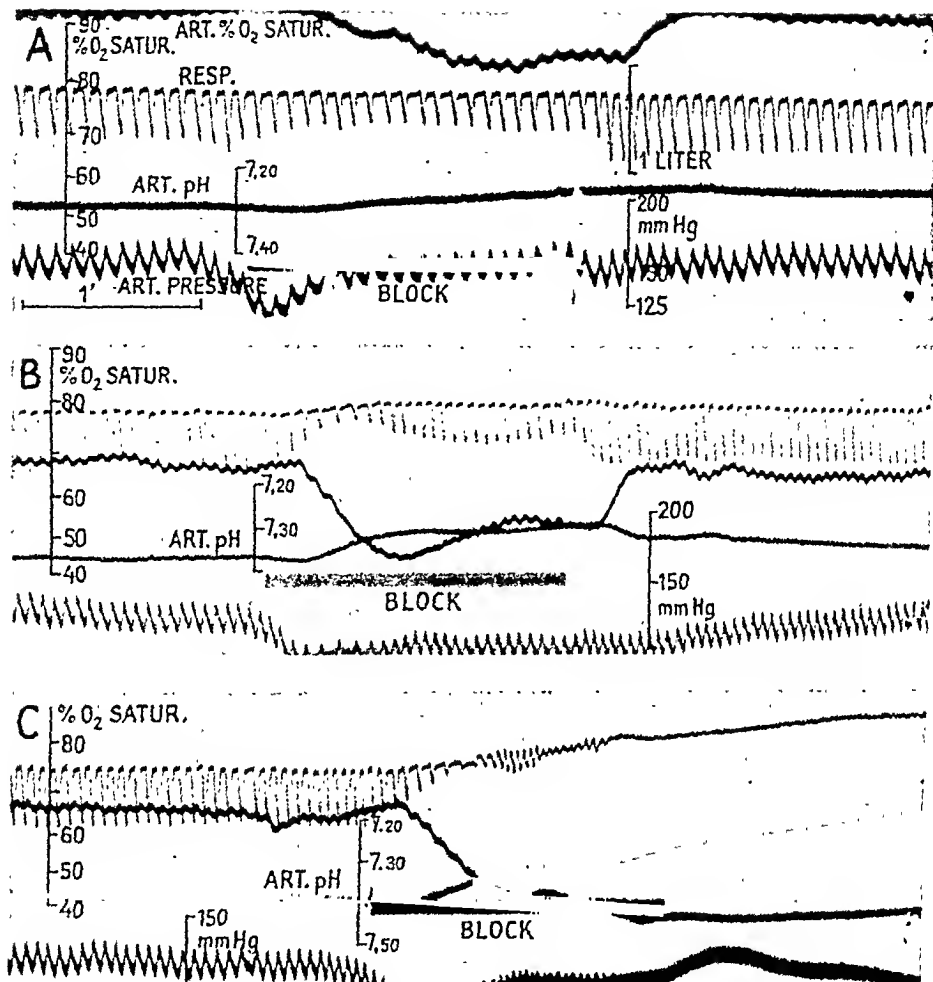


Fig. 9. The figures show the decrease of the centrogenic support from its eupneic amount during the development of the respiratory alkalosis, as revealed by withdrawal of the reflexogenic component. The moments of blocking the chemoreceptor signals essentially correspond to points A, B and C in fig. 8.

A. Room air. Note importance of the chemoreflex drive for the maintenance of the eupneic arterial O<sub>2</sub> saturation under anaesthesia as revealed by its withdrawal ('Block').

B. After 5 minutes breathing of 8.2 % O<sub>2</sub> in N<sub>2</sub>. Note that the secondary increase of the centrogenic support during the blocking runs parallel to the arterial pH.

C. After 20 minutes breathing of 8.2 % O<sub>2</sub> in N<sub>2</sub>. Note that the secondary increase of the centrogenic support during the blocking was here insufficient in raising the arterial O<sub>2</sub> saturation, because the center became "paralyzed" by hypoxemia before the increasing arterial pH could stimulate the center again. Therefore the center ceased to respond to increasing arterial pH as well as to chemoreflex influence, when the latter was re-established by deblocking. The animal was later resuscitated by artificial ventilation.

of the centrogenic support to the ventilation in oxygen deficiency is also due to a devitalizing effect of the hypoxemic state upon the center, as is the case when the effect of oxygen deficiency is tested after the chemoreflexes have been permanently disconnected. Evidence, however, points against such an interpretation of the decreased centrogenic support in acute hypoxic hyperventilation. The negative arguments are: 1) If the blocking of the chemoreflexes during acute hypoxic hyperventilation was sufficiently prolonged the respiratory activity would again increase somewhat before it completely subsided, even if sudden apnea was caused by the blocking (figs 9 B and C). There would not have been such a secondary increase of the centrogenic breathing during the period of blocking if the chemosensory cells of the centre had become paralyzed by the preceding hypoxemia. This paralysis should instead have increased as a result of the rapidly increasing oxygen unsaturation when the breathing decreased, and should have terminated in death without a secondary increase in the respiratory activity. 2) The continuous recording of the pH of the circulating arterial blood revealed the center's capability to respond to the increase of the arterial cH, that occurred during the respiratory insufficiency under sinus nerve block (fig. 9 B). In cases of apnea during prolonged sinus nerve block the apnea was usually broken as the arterial cH increased. Fig. 9 C shows that the secondary increase of the centrogenic breathing may be insufficient to keep the arterial oxygen saturation high enough to retain the response of the center to increasing arterial cH. The center was here devitalized by the increasing hypoxemia during the blocking, not by the degree of hypoxemia before the blocking. 3) The blocking effect was closely related to the degree of arterial alkalosis, produced by the hypoxic hyperventilation. It was observed that the blocking effect was greater, the greater the alkalosis. The centrogenic support was not only less in acute hypoxic hyperventilation than in eupnea, but became even smaller as arterial alkalosis became more severe. This effect was first obtained by inducing ambient oxygen deficiency of varying degrees. It was found that the centrogenic support became less, when the ambient oxygen deficiency increased and thus the hyperventilation and the arterial alkalosis increased. To this interpretation it might perhaps be objected that the increased hypoxemia per se might have been a contributing factor towards decreased centrogenic support. However, evidence against this objection was

obtained as follows. If the decrease of the centrogenic support during acute hypoxic hyperventilation is due to the devitalizing effect of hypoxemia and not to the respiratory alkalosis, there should always be the same or a smaller centrogenic breathing after a period of acute hypoxic hyperventilation than in the initial stage when the hypoxemia had exerted its influence during a shorter period of time. Fig. 10 shows, however, that the effect of blocking is less after the longer period of constant degree of arterial oxygen unsaturation if at the same time the arterial  $\text{cH}$  is higher. This finding is in complete harmony with the author's opinion that the decrease of the centrogenic support during hypoxic hyperventilation is due to the respiratory alkalosis. Devitalization of the centres by the hypoxemia per se is not excluded as a contributing cause, but is conceivably of very little importance. It was also found that even after prolonged blockings, producing a severe degree of arterial oxygen unsaturation, the volume of breathing immediately increased again on deblocking, and that this re-establishment of the chemoreflex drive would usually set up a volume of breathing, that was greater than before the blocking (fig. 9 B). This argues against decreased vitality of the centres. The minor importance of the hypoxemia per se for the decrease of the centrogenic drive in acute hypoxic hyperventilation is shown by fig. 11. In this case the inhaled oxygen percentage was suddenly changed from 8.2 to 20 at the moment when the block was applied, so that the arterial oxygen saturation was high when the block took effect. In spite of the high arterial oxygen saturation the center remained apneic.

It was mentioned above that the experiments of the present work showed that the centrogenic support is not only less in the alkalotic stage of acute hypoxic hyperventilation than in eupnea, but that a more severe blood alkalosis from increased ambient oxygen deficiency decreases the centrogenic support still more. One may now inquire, whether the gradually developing respiratory alkalosis during a constant degree of reduced ambient oxygen pressure causes a gradual decrease of the centrogenic drive, although there is continuous hyperventilation. In other words: does the centrogenic drive decrease during the increasing respiratory alkalosis, that develops when the organism is subjected to a constant degree of oxygen deficiency in the inspired air? It has been pointed out above that in the acute stage of hypoxic hyperventilation the resulting blood alkalosis only gradually reaches its maximum.

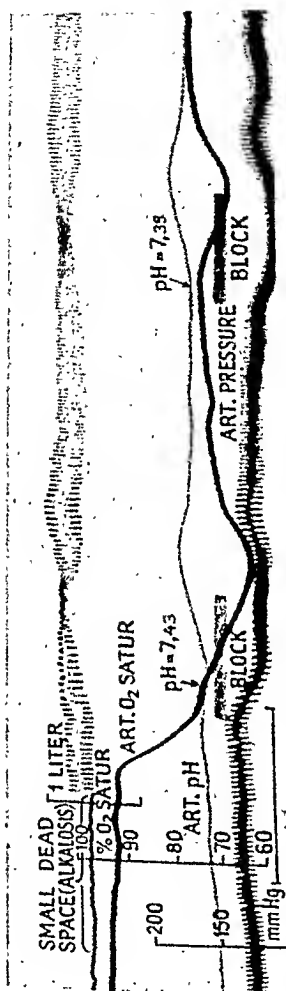


Fig. 10. The figure shows that the eutrogenic support, revealed by withdrawal of reflexogenic component, increases in the aceto stage of oxygen deficiency, if the arterial  $\text{CH}$  is artificially made to increase in stand of decrease. At the second period of blocking, the eutrogenic support was greater in spite of the fact that hypoxemia per se had been acting upon the center for a longer period of time. The increase of the arterial  $\text{CH}$  was induced by first having the dog breathe room air with a diminished dead space, causing a  $\text{CH}$  decrease corresponding to an increase of 0.1 of a pH unit. The recording of the volume of breathing began with inhalation of 8.2 %  $\text{O}_2$  in  $\text{N}_2$  at the same time the dead space was again increased. See text p. 64. Time working = 1 minute.

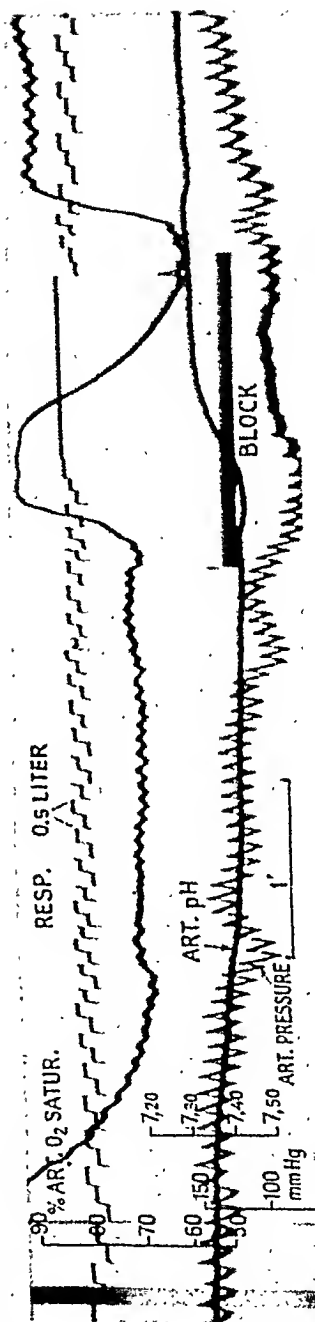


Fig. 11. The figure demonstrates the minor significance of the hypoxemia per se for the failure of the eutrogenic support in hypoxic hyperventilation. 8.2 %  $\text{O}_2$  was substituted for room air at the double marking. When hypoxic hyperventilation was established, the chemoreceptor signals were blocked a few seconds after the dog had suddenly been made to breathe room air again. The blocking revealed that the center remained apneic in spite of the great increase in the arterial  $\text{O}_2$  saturation, which was thus induced. The arterial oxygen saturation decreased again during the blocking as a result of the apnea but increased greatly on deblocking, when the dog resumed breathing of room air.

Repeated short blockings of the chemoreflexes during the increase of the respiratory alkalosis in acute hypoxic hyperventilation (at points B and C, fig. 8) clearly demonstrated that the centrogenic support decreases in close relationship to the increasing alkalosis in the arterial blood. Whereas the centrogenic breathing was less than in eupnea during the initial stage of hypoxic hyperventilation, before the respiratory alkalosis had reached its maximum, the same degree of ambient oxygen deficiency would cause centrogenic apnea when the alkalotic change had become more pronounced (figs 9 A, B and C).

Summing up the above the following can be said of the rôle of the respiratory center during the acute stage of the hyperventilation, that results from a constant degree of oxygen deficiency in the inspired air. At the moment when the hyperventilation just begins the centrogenic drive is about the same as in eupnea. During the development of respiratory alkalosis the centrogenic support gradually diminishes in a close relationship to the increase of the respiratory alkalosis. At the maximum of respiratory alkalosis the centrogenic drive may be zero if the ambient oxygen deficiency is of such degree that the actual degree of alkalosis is severe enough. Hence, the chemosensitive cells of the centres have no share in the increase of the respiratory activity. Evidence has been presented, that the continuous decrease in the self-engendered activity of the chemosensitive cells of the centres in acute hypoxic hyperventilation is due to the increasing respiratory alkalosis, that is caused by centrogenic drive itself plus the chemoreflex drive, *i. e.* the resultant hyperventilation. This evidence has been obtained by a correlation between the simultaneous changes in the volume of breathing, the arterial oxygen saturation and the arterial pH during and after the blocking of the afferent chemosensory impulses in acute hypoxic hyperventilation. The evidence presented here refutes those earlier theories which maintain that, in tolerated oxygen deficiency with the chemoreflex mechanism intact, the center becomes more acid from local accumulation of lactic acid or that the vegetative functions of the centres are significantly inhibited by the hypoxemia *per se*. It is obvious, that as far as the self-engendered activity of the chemosensitive cells of the center is concerned, this activity stands in no paradoxical relationship to the alkaline change in the arterial blood during acute hypoxic hyperventilation. For the center itself contributes less to the acute hypoxic hyper-

ventilation than to eupneic breathing, and this is simply because its own chemosensitive cells are not stimulated by the actual arterial  $p\text{CO}_2$  or  $\text{cH}$  as much in acute hypoxic hyperventilation as in eupneic breathing. The results described in fact support the inference that in acute hypoxic hyperventilation the centre turns alkaline as does the arterial blood.

Thus as far as the center is concerned, the first "reaction theory" of WINTERSTEIN (cf. p. 19), according to which the activity of the center should be controlled by the reaction of the blood even in acute hypoxic hyperventilation, has gained a certain experimental support by direct experimental approach. The revival of the theory can, however, only be partial. Two main defects in the theory remain, for acute oxygen deficiency does not induce blood acidosis, and the acute hypoxic hyperventilation is not controlled by the chemosensitive cells of the center itself.

On the other hand, the second "reaction theory" of WINTERSTEIN (cf. p. 19) — or the cellular acidity theory of GESELL — according to which the center should turn acid in acute, tolerated oxygen deficiency, as opposed to the alkaline change in the arterial blood, has not been supported by the present experiments. From the latter it may be assumed that the center becomes more alkaline than it is during eupnea.

b) *The Chemoreceptors and the Outcome of Chemoreceptor Activity: the Chemoreflex Drive.*

It is evident from the above, that, during the acute stage of the hypoxic hyperventilation, resulting from a constant degree of oxygen deficiency, the centrogenic support decreases in close relationship to the increasing respiratory alkalosis of the arterial blood. Hence, it is obvious that as far as the centre is concerned, the hypoxic hyperventilation tends to counteract itself during the stage of increasing alkalosis.

The foregoing results concerning the respiratory center thus gives no explanation of the paradox of acute hypoxic hyperventilation. The explanation must be elsewhere. In the following it will be shown, that the paradox is entirely restricted to the share of the chemoreflex drive.

As can be anticipated from the preceding experiments, the chemoreflex drive must not only increase in order that hyperventilation be set up, but increase in such degree as to afford com-

pensation also for the gradual decrease of the centrogenic support. This was demonstrated by repeated blockings during the development of the respiratory alkalosis. The blockings cut off an increasing part of the hyperventilation as the alkalosis developed (figs. 9 and 12).

It was furthermore found (figs. 11, 12 and 13) that although the arterial oxygen saturation relatively rapidly fell to its lowest level (the ambient oxygen deficiency remaining constant) the volume of breathing continued increasing for a certain period of time after this level was attained, in spite of the fact that the arterial  $cH$  was also continuously decreasing. The arterial oxygen saturation would even rise somewhat again, sometimes quite considerably (fig. 12). Now, from the present knowledge of the physiologically active factors, that stimulate the chemoreceptors and the center, one would not expect that a constant or actually decreased degree of arterial unsaturation would run parallel with a continuing increase of the respiratory activity, if at the same time the arterial  $cH$  decreases. The obscure increase in the respiratory activity must refer to the chemoreflex drive, since it has been shown that the centrogenic component decreases with the arterial  $cH$ . The question arises, whether this continuing increase of the chemoreflex drive is due to increased chemoreceptor stimulation or to increased outcome of stimulation. Theoretically, a constant firing activity of the chemoreceptors may, of course, have different influence upon the activity of the center under different circumstances, be the change caused by chemical modifications at the center or by other mechanisms.

Preliminarily it is obvious, that if increased stimulation of the chemoreceptors was the cause of the continuing increase of the chemoreflex drive, it can conceivably not have been due to the stimulus of arterial  $pCO_2$  or  $cH$ , since increasing alkalosis prevailed. However, the hypoxemic stimulus to the chemoreceptors may in certain cases have increased, although the arterial oxygen saturation remained constant or even increased somewhat. Constant arterial oxygen unsaturation plus increasing alkalosis causes a decrease in the arterial oxygen pressure (calculating the  $pO_2$  from the determined  $pH$  and oxygen saturation and a standard dissociation curve), and increased stimulation of the chemoreceptors by decreased arterial oxygen pressure is, therefore, theoretically possible. That a decreasing arterial oxygen pressure can not be entirely responsible for the paradoxical increase of the chemo-

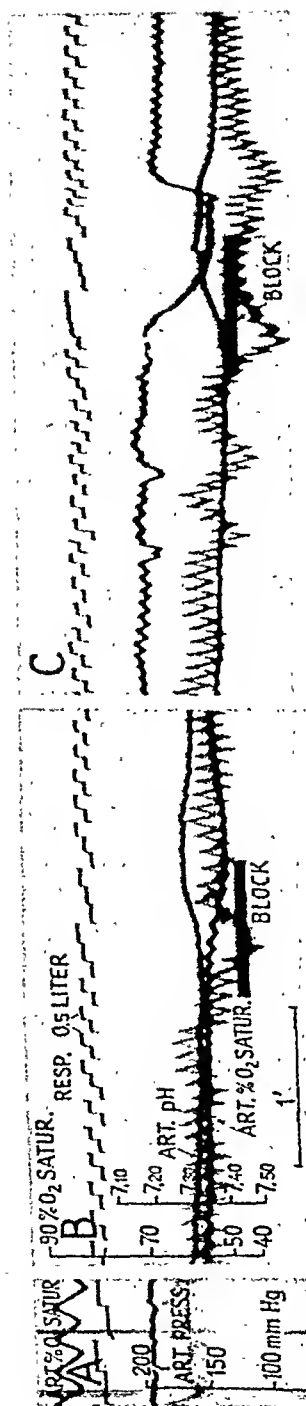


Fig. 12. Effects of withdrawing the reflexogenic component in two different stages of acute hypoxic hyperventilation. The blocking cut off a much greater part (= chemoreflex drive) of the volume of breathing in C than in B, in spite of the fact that the hypoxemic stimulus to the chemoreceptors must have been greater in B than in C. Note that the respiratory alkalosis was more severe in C than in B. See text p. 68.

A. Breathing room air. — B. After 5 minutes' breathing of 6.7 %  $O_2$  in  $N_2$ . — C. 20 minutes later.



reflex drive during increasing alkalosis is, however, obvious from the fact that the relationship between the arterial oxygen saturation and the actual alkalosis often did not allow the assumption of a decreased arterial oxygen pressure. This was especially striking in those cases, where the arterial oxygen saturation again rose considerably after first having attained a lower level. Thus fig. 12 shows 1) that the arterial oxygen saturation may again rise considerably during the course of acute hypoxic hyperventilation, 2) that the chemoreflex drive may increase significantly in spite of the decreased arterial  $cH$  and in spite of the secondary increase of the arterial oxygen saturation and 3) that decreased arterial oxygen pressure can not have been the cause of the increase of the chemoreflex drive. For the decrease in the arterial  $cH$  can not have caused the arterial pressure to be lower in fig. 12 C than in fig. 12 B.

The question whether chemical modification at the center or along the chemoreflex nervous pathways may actually exaggerate the outcome of the chemoreceptor activity is of special interest. Such an intermediary mechanism was surmised by GEsELL, LAPIDES & LEVIN (1940). They found that if the chemoreflexes were cold-blocked during hypercapnic hyperpnea the chemoreflex drive proved to be insignificant and actually less than in eupnea. It was therefore cursorily proposed that hypocapnia, such as occurs in acute hypoxic hyperventilation, might lead to a progressively increasing potentiation of the outcome of the chemoreflex signals, thereby assuring an increasing dominance of the chemoreflex drive. No direct evidence was, however, presented.

From the above-mentioned result of the present work, that the chemoreflex drive increases as the respiratory alkalosis increases, although the chemoreceptor excitation can hardly be assumed to increase, it seems highly probable, that the increase of the chemoreflex drive is in some way connected with the increasing alkalosis. The assumption was directly tested by observing the magnitude of the hypoxic hyperventilation, when the tendency to increasing alkalosis was suppressed by the addition of carbon dioxide to the inspired air (fig. 13). The dog first breathed a mixture of 8.2 per cent oxygen in nitrogen, thereby developing a considerable hyperventilation with respiratory alkalosis (fig. 13 B). Then the oxygen content was diminished, and carbon dioxide added to the inspired air. By such an arrangement it was intended to decrease the alkalosis and to keep the arterial oxygen saturation

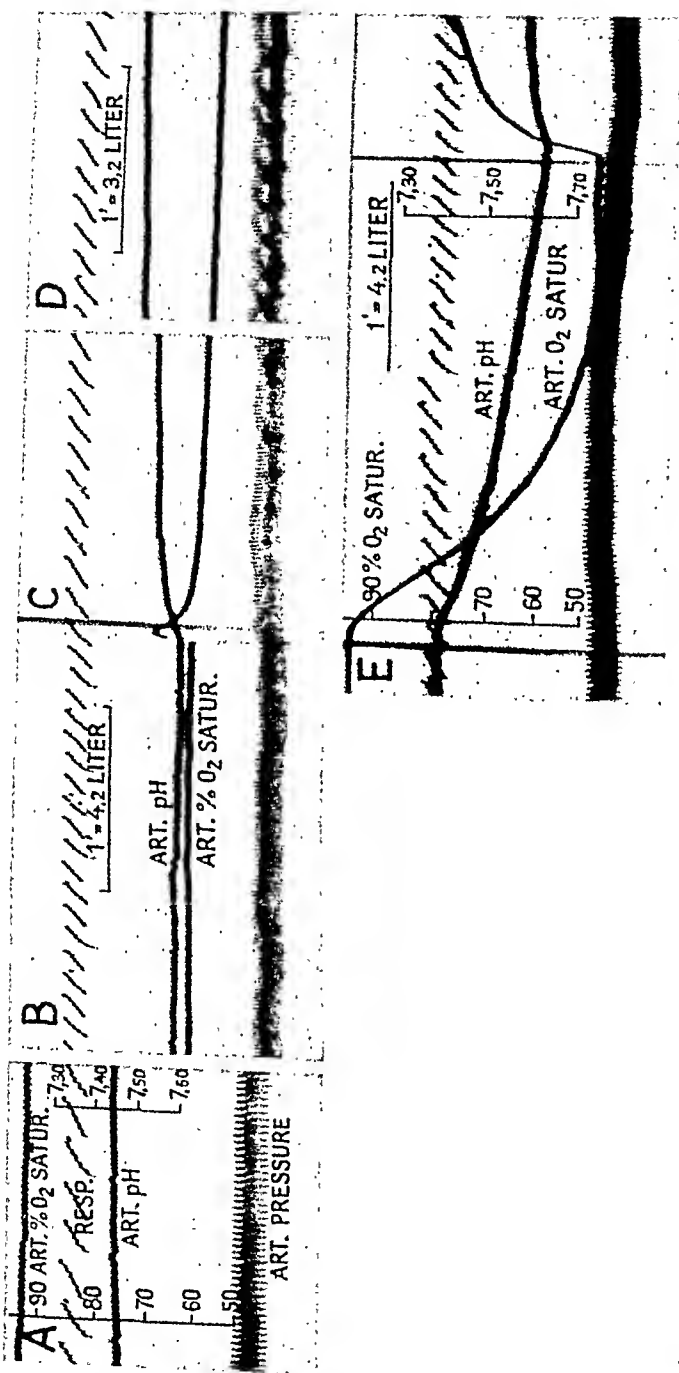


Fig. 13. Effect upon magnitude of hypoxic hyperventilation from suppression of respiratory alkalosis. (Calibrations in A refer to A, B, C, and D. Arterial pressure = 130–140 mm Hg.)

A. Room air. — B. After 3 minutes' breathing of 8.2 % O<sub>2</sub> in N<sub>2</sub>. — C. Inhalation of expired gas, collected during preceding breathing of 8.2 % O<sub>2</sub> in N<sub>2</sub> (diminished O<sub>2</sub> content, increased CO<sub>2</sub> content in order to reduce respiratory alkalosis and arterial O<sub>2</sub> saturation. The hump in the O<sub>2</sub> saturation curve is due to an incidental breath of room air). Note diminished hyperventilation. — D. 2 minutes later. — E. Shift from room air to 6.7 % O<sub>2</sub> in N<sub>2</sub> and back to room air, at markings. (For further explanation, see text p. 70.)

low enough to avoid contamination with the effect of decreasing alkalosis upon the arterial oxygen pressure. Fig. 13 C and D shows that the volume of breathing now distinctly decreased as the arterial cH increased. The arterial oxygen saturation thereby decreased to a level considerably below that attained in the case of hypoxic hyperventilation with unhampered alkalosis. In spite of the increase of the hypoxemic stimulus to the chemoreceptors that conceivably must have occurred, and in spite of the probable stimulation of the center and chemoreceptors by the increase in the arterial cH, the volume of breathing was less than in the case of unhampered respiratory alkalosis. The degree of hypoxemia in the case of "suppressed alkalosis" could not per se have caused depression of the respiratory apparatus, for immediately afterwards the animal responded to 6.7 per cent of oxygen with vigorous hyperventilation, although the arterial oxygen unsaturation here became much more severe (fig. 13 E). The obvious inference is that, if the development of respiratory alkalosis is suppressed during acute hypoxic hyperventilation, the full development of the hypoxic hyperventilation may also be suppressed. It is hard to explain such a mechanism except in terms of a decreased chemoreflex drive, caused by the suppression of the alkalosis.

It should be emphasized here that this is of course not meant to imply that inhalation of carbon dioxide will counteract hyperventilation in conditions of slight hypoxemia (with slight respiratory alkalosis), hypercapnic hyperpnea or metabolic acidosis. The inhibitory action of (relatively) increased arterial  $p\text{CO}_2$  or cH only refers to conditions in which hyperventilation is mainly or entirely maintained by the chemoreflex drive, *e. g.* conditions of acute hypoxemia, moderate or severe. There remains to be investigated at which point of arterial oxygen saturation (or pressure) the stimulating effect of carbon dioxide just counterbalances its inhibitory effect upon the chemoreflex drive.

Even a small increase of the dead space should accordingly counteract the full development of the chemoreflex drive, an inference that should have a certain bearing upon the designs of oxygen masks for flying at altitudes above 30,000 feet. The contested advisability of adding carbon dioxide to the inspired air during flying at high altitudes may perhaps require reconsideration in view of the results of the present experiments.

Summing up the evidence obtained on the chemoreflex drive

in hypoxic hyperventilation during the stage of developing respiratory alkalosis, the following can be said. The acute hypoxic hyperventilation is entirely caused by the chemoreflex drive, originally initiated by hypoxemic chemoreceptor excitation. As the respiratory alkalosis develops the chemoreflex drive necessarily becomes increasingly important, on account of the continuous decrease in the centrogenic support. In the stage of maximum respiratory alkalosis, hypoxic hyperventilation may be entirely maintained by the chemoreflexes, the chemosensitive cells of the center being apneic from missing stimulation. Evidence has been presented that the chemoreflex drive is not only determined by the prevailing degree of hypoxemia, but is actually potentiated by the respiratory alkalosis. It is possible that the potentiating effect of alkalosis is due to synaptic alkalization, but other points of attack along the chemoreflex pathway may also exist. At present no conclusion can be drawn on this subject. From the teleological point of view, however, the reflex character of the connections between the central and peripheral chemosensitive cells is significant with respect to the demonstrated importance of a chemical intermediary mechanism, modifying the outcome of chemoreceptor excitation (cf. GESELL, LAPIDES & LEVIN 1940).

#### **Prolonged Oxygen Deficiency: the Effect of the Compensation of Respiratory Alkalosis upon the Balance of Components in the Respiratory Control.**

In the definition of the problem it was pointed out that information has been lacking not only on the interaction of centrogenic and chemoreflex components during the development of the respiratory alkalosis in the acute stage of constant oxygen deficiency, but also on the importance of the chemoreflex drive for the hypoxic hyperventilation in later stages of the same degree of ambient oxygen deficiency. It seems to be pertinent to inquire, whether the unbalance between the centrogenic and chemoreflex drives, that has been shown to develop during the acute stage of hypoxic hyperventilation, is retained if the respiratory alkalosis is allowed to become compensated spontaneously.

For this purpose blocking experiments were made during hypoxic hyperventilation after the animals had been exposed to 8—11 per cent of oxygen at atmospheric pressure for 6—10 hours. It was observed that a very small or no effect upon the volume of breathing was obtained. The chemoreflex drive had diminished or

completely subsided, and the hypoxic hyperventilation was thus mainly or entirely maintained by the chemosensitive cells of the center itself. It was possible to test the reversibility of the chemoreflex drive in one case because of the good condition of the animal. In this case the chemoreflex drive was found to be completely absent after an exposure of 5 hours to 8.2 per cent of oxygen. After a subsequent exposure of two hours to 100 per cent of oxygen, the effect of blocking the chemoreflexes was again observed under the acute influence of 8.2 per cent of oxygen. The blocking now revealed a return of the chemoreflex drive, giving approximately the maximum effect that had been obtained in the beginning of the prolonged exposure.

It is possible, although not probable, that in the blocking experiments after prolonged exposure to oxygen deficiency, in which the reversibility of the chemoreflex drive was not tested, the chemoreceptors or the chemoreceptive fibers might have been damaged during the prolonged experiment, the ceasing of the chemoreflex drive thus partly being due to an artifact. However, the fact that undiminished hyperventilation continued seems to indicate at least, that the share of the center in the hypoxic hyperventilation greatly increases as the alkalosis becomes compensated during the course of a prolonged exposure to constant ambient oxygen deficiency.

From the discussion of the interaction of centrogenic and chemoreflex control of breathing in the stage of developing respiratory alkalosis, it is obvious that this change in the relationship between the centrogenic and chemoreflex components could actually be anticipated. The finding of decreased chemoreflex and increased centrogenic drives is in complete harmony with the conception previously advanced, *i. e.* that the outcome of the chemoreceptor excitation is exaggerated by alkalosis and diminished if the alkalosis is reduced, and that the centrogenic support essentially runs parallel with the changes in the acidity of the chemosensitive cells of the center (this acidity approximating to the acidity of the arterial blood). The fact that during prolonged exposure to oxygen deficiency the center appeared gradually to regain the upper hand of the chemoreflexes, thereby becoming more or less solely responsible for the hypoxic hyperventilation as normal arterial reaction returns is, accordingly, not astonishing.

It is significant, that in the stage of hypoxic hyperventilation when the compensation of the respiratory alkalosis had become

nearly complete, the centrogenic component was actually greater than previously during eupnea. The arterial pH was, however, not more acid than it had been during eupnea, but instead slightly more alkaline. That the centrogenic component was supernormal may at first seem inconsistent with the conception, that the self-engendered activity is essentially controlled by the reaction of the arterial blood (or by the reaction within the center itself). But, remembering that hyperventilation must be combined with a decrease of the arterial  $p\text{CO}_2$  and that the arterial  $\text{cH}$  had nearly returned to its eupneic value, it follows from the Henderson-Hasselbalch equation that the arterial  $\text{BHCO}_3$  concentration must also have been less than it was during eupnea. Hence, provided the production of metabolic carbon dioxide remains constant, supernormal centrogenic breathing must go on, in order that the  $\text{cH}$  in the center or in the arterial blood shall not increase above its eupneic level. As has been pointed out before, the conception that the chemosensitive cells of the center respond to changes in acidity does not imply that a certain pH shall induce a certain volume of centrogenic breathing.

The change in the mechanism of hypoxic hyperventilation, which has thus been shown to occur during the course of a prolonged exposure to constant ambient oxygen deficiency may, accordingly, be expressed as follows. Whereas the pulmonary ventilation is mainly controlled by the need for oxygen in the acute stage of hypoxic hyperventilation (drive = essentially reflexogenic), the same or nearly the same amount of ventilation is essentially maintained by the need for carbon dioxide removal as the compensation of the respiratory alkalosis becomes complete (drive = mainly centrogenic).

This interpretation of the finding of diminished or lacking effect of blocking the chemoreflexes after prolonged exposure to oxygen deficiency has been tested by observing the effect of a sudden increase in the oxygen supply at points C and D, fig. 8. In these cases the vagi were not sectioned, and the carotid regions were completely intact. Accidental damage to the chemoreceptors is therefore excluded. It was observed, that a sudden administration of room air during hypoxic hyperventilation in the stage of maximum respiratory alkalosis (point C, fig. 8), would immediately decrease the ventilation. The new volume was markedly insufficient, for it took 5 to 20 minutes for the arterial oxygen saturation to reach its prehypoxic level. In certain

cases apnea ensued as soon as the arterial oxygen saturation had increased only insignificantly, and the arterial oxygen saturation thereby fell again, sometimes even below the lowest level it had attained under the preceding influence of oxygen deficiency (fig. 14 B). This paradoxical insufficiency of breathing after increased oxygen supply in the alkalotic stage of hypoxic hyperventilation may be explained as follows. From the preceding discussion concerning the interaction of centrogenic and chemoreflex drives during the acute stage of hypoxic hyperventilation (developing respiratory alkalosis) it is apparent that the insufficiency of breathing after a sudden increase in the oxygen supply may be due 1) to decreased stimulation of the center from the lingering alkalosis, and 2) to decreased stimulation of the chemoreceptors from the rise in the arterial oxygen saturation that is caused by the increased oxygen supply. If the preceding oxygen deficiency has been effective in developing a considerable alkalosis, the hyperventilation may be entirely reflexogenic at the moment when the rise in the arterial oxygen saturation sets in, the chemosensitive cells of the center itself being apneic. It is obvious then, that, since no centrogenic support is active in the respiratory activity, a rise in the arterial oxygen saturation from suddenly increased oxygen supply will produce a dangerous condition, a condition that is characterized by a missing stimulation of the chemosensitive cells of the center and by a decreased stimulation also of the chemoreceptors. In the stage immediately following the increase in the oxygen supply, the respiratory activity will, therefore, eventually be maintained only by the chemoreflex drive, and this drive is considerably diminished by the increase in the arterial oxygen saturation. As was mentioned above, apnea would sometimes develop as soon as the arterial oxygen saturation had only increased insignificantly, and the arterial oxygen saturation would thereby temporarily fall again and, actually, below the level, that had previously maintained hypoxic hyperventilation (fig. 14 B). This result may at first seem difficult to explain, especially since the greatly increasing arterial  $cH$  during this apnea might be expected to stimulate the center. The finding may, however, be considered as further evidence that the outcome of chemoreceptor activity is greatly influenced by reaction of the arterial blood. For it seems most probable that the increase in the chemoreceptor excitation, that must necessarily have occurred during the period of apnea, was effectively blocked by the in-

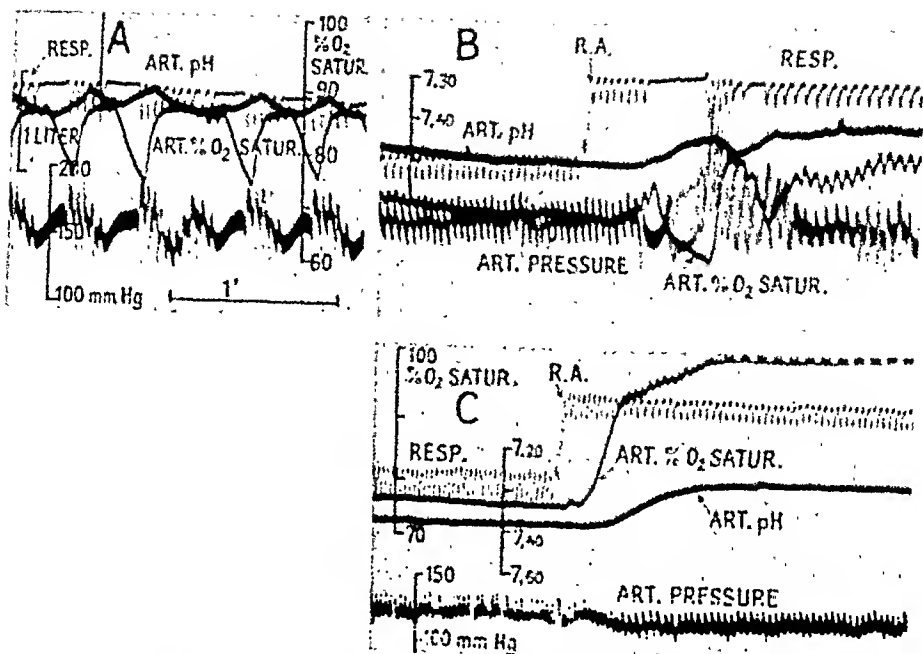


Fig. 14. Effects of a sudden increase in the oxygen supply during hypoxic hyperventilation at the stage of maximum respiratory alkalosis (point C, fig. 8) and after the compensation of the alkalosis has become nearly complete (point D, fig. 8). The calibrations in A and B apply to both figures.

A. Periodic breathing in room air.

B. After breathing of 8.2 % O<sub>2</sub> in N<sub>2</sub> for 15 minutes. At R. A. sudden supply to the spirometer of O<sub>2</sub> so that its O<sub>2</sub> content became rapidly the same as in room air. Note insufficiency of breathing, and the first apnea (cf. p. 76).

C. After 8 hours' breathing of 8.2 % O<sub>2</sub> in N<sub>2</sub>. The respiratory alkalosis was now nearly compensated. R. A.: see B. Note the rapid and great increase in the arterial O<sub>2</sub> saturation, compared to B.



creasing arterial  $cH$ , this increase in the arterial  $cH$ , however, not being able to stimulate the chemosensitive cells of the center because the arterial  $cH$  was still alkaline. Therefore, apnea lasted till a moment, when the arterial oxygen saturation was considerably less and the arterial  $cH$  considerably higher than was the case during the preceding actual hypoxic hyperventilation. In other words: during the apnea that may follow upon a sudden increase in the ambient oxygen pressure during hypoxic hyperventilation and maximum respiratory alkalosis, the arterial  $cH$ , although increasing, may not be high enough to stimulate the center, but may become high enough to block the outcome of the increased excitation of the chemoreceptors that results from the decrease in the arterial oxygen saturation.

From the above it is obvious, that a sudden increase in the oxygen supply during severe respiratory alkalosis from oxygen deficiency in the inspired air, may be particularly dangerous to the body in a paradoxical way. For increased oxygen supply may under such circumstances actually cause a decrease in the arterial oxygen saturation from respiratory insufficiency. The finding may possibly explain some of the deleterious effects that sometimes occur, when aviators during breathing of rarefied air at high altitudes suddenly resume oxygen inhalation. Such cases (some combined with actual casualties) have been reported by SCHWARTZ & MALIKIOSIS (1938), JOKL (see SCHMIDT & COMROE 1941) and SCHMIDT & COMROE (1941). SCHMIDT & COMROE have put forth the question (1941), whether these situations may be similar in some respects to that existing in those patients who die when a severe tracheal obstruction is suddenly relieved by tracheotomy. In such cases alkalosis is not likely to prevail, however, but rather respiratory acidosis from respiratory depression. In these cases, it is probable, therefore, that, when the arterial oxygen saturation suddenly increases, the chemoreflex drive suddenly becomes zero, partly because of the decrease in the stimulation of the chemoreceptors and partly because the increasing acidosis (from the resulting respiratory standstill) hampers the outcome of the chemoreceptor excitation, that must sooner or later occur as the arterial oxygen saturation again decreases.

The finding of respiratory insufficiency following a sudden increase in the oxygen supply during hypoxic hyperventilation in the stage of maximum respiratory alkalosis has also a certain bearing upon some anaesthesiological questions. It is obvious

that care must be taken, not to increase the oxygen supply suddenly, if there is any reason for the assumption that the ventilation is maintained entirely by the chemoreflex drive. Such a condition may easily occur if for any reason oxygen deficiency is present. Especially in profound narcosis the respiratory activity may be maintained entirely by the chemoreflex drive, resulting from a certain degree of arterial oxygen unsaturation and from depression of the respiratory center by the anesthetic used. BENZINGER, OPITZ & SCHOEDEL (1939) report an apnea of 10 minutes following a sudden increase in the oxygen supply from room air to 100 per cent in a dog under deep Pernoxton anaesthesia. From the foregoing it is most probable, that this apnea was primarily due to decreased chemoreceptor excitation and to depression of the centrogenic component by the anaesthesia, and secondarily to the increasing acidosis, that must inevitably have developed during the apnea, causing a blocking of the chemoreceptor signals, which must as inevitably have increased again during the apnea. Otherwise the decreased arterial oxygen saturation would be expected to have initiated breathing much sooner.

Further evidence, that the balance of the centrogenic and chemoreflex components changes during hypoxic hyperventilation as the respiratory alkalosis spontaneously becomes compensated, was obtained, when the effects of a sudden increase in the oxygen supply during maximum respiratory alkalosis (point C, fig. 8) were compared with those effects, which occurred at point D (fig. 8) in the same animals. Although room air was administered at both points, the decrease in the respiratory activity was much less when the respiratory alkalosis had nearly become compensated, than at the point of maximum respiratory alkalosis. The difference was strikingly illustrated by the abrupt and great increase in the arterial oxygen saturation, which now occurred as soon as room air was administered. Generally the arterial oxygen saturation rose to a level higher than was originally maintained during eupneic breathing, *i. e.* before hypoxic hyperventilation prevailed (fig. 13 A and C). That the respiratory activity was here maintained at a greater magnitude after increased oxygen supply, than when the effect was observed in the stage of maximum respiratory alkalosis, is also obvious from the fact that the arterial  $\text{cH}$  rose to about the same degree as in the stage of maximum alkalosis, thereby overshooting the original eupneic level. For this

increase of arterial  $cH$  means a smaller increase in the arterial  $pCO_2$  (smaller decrease in the pulmonary ventilation), since the arterial  $BHCO_3$  concentration must necessarily have been less here than during the stage of maximum respiratory alkalosis.

This finding of a smaller decrease of the volume of breathing when room air was administered after prolonged exposure to a constant degree of ambient oxygen deficiency instead of in the acute stage, illustrates the "fixed hyperventilation" (cf. p. 35). The minor influence of a sudden increase in the oxygen supply after prolonged exposure upon the volume of breathing, is in complete harmony with the results of the blocking experiments described above. A fixation of the hyperventilation may, in fact, be anticipated, when remembering that an increased share of the chemosensitive cells of the center itself in this stage of hypoxic hyperventilation was demonstrated. The centrogenic component here maintains the greatest part of the hyperventilation, and a diminished excitation of the chemoreceptors, occurring as the arterial oxygen saturation increases during the increased oxygen supply, should accordingly only have a minor influence upon the volume of breathing. That the chemoreflexes continue to play a certain rôle for the hypoxic hyperventilation, even after prolonged exposure to oxygen deficiency is, however, likely, since the respiratory activity actually decreased somewhat.

That the centrogenic breathing becomes supernormal after prolonged exposure to oxygen deficiency does not mean that the center should gain the ability of being stimulated by oxygen lack. The supernormal centrogenic breathing is maintained by the need for carbon dioxide removal, as is the case in eupneic breathing, *i. e.* the center reacts to changes in the acidity of the arterial blood or within its own cells. That the centrogenic breathing is supernormal can be assumed to depend upon the decrease of the buffering capacity of the blood or of the center's own intracellular fluids against carbon dioxide of metabolic origin.

The *deus ex machina* in the change of the interaction of centrogenic and chemoreflex control of breathing at rest during the course of a prolonged exposure to a constant degree of tolerated oxygen deficiency in the inspired air seems to be the chemoreflex drive. The increase of the chemoreflex drive in the acute stage of oxygen deficiency protects the body from the deleterious effect of want of oxygen, thus constituting an urgency mechanism primarily. This mechanism is, however, not meant to retain its

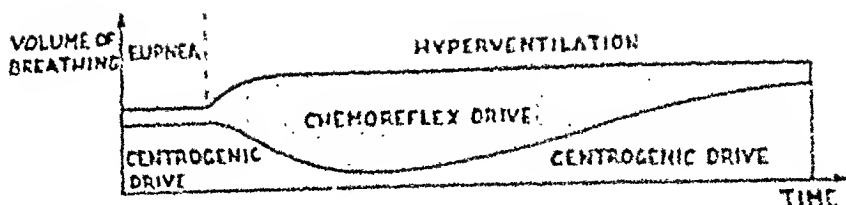


Fig. 15. A highly schematic diagram illustrating how the interaction of ventilogenic and chemoreflex components in breathing changes, when exposure is transferred to hyperventilation by a rapidly induced, constant degree of ambient oxygen deficiency. In the acute stage of hypoxic hyperventilation (developing respiratory alkalosis) the chemoreflex drive becomes increasingly more important, initiated by the hypoxic stimulus and potentiated by the alkalosis. The centrogenic support decreases in relation to the increasing alkalosis. After prolonged exposure, when spontaneous compensation of the respiratory alkalosis has become more or less complete, the hyperventilation is maintained mainly by the chemoreflex support, which has again gained the upper hand of the chemoreflexes. The centrogenic support is now greater than in rapid.

dominance over the centrogenic component if the influence of ambient oxygen deficiency is prolonged, as in dwellers at high altitudes. As acclimatization to a certain degree of ambient oxygen deficiency develops, the center again gains the upper hand of the chemoreflex drive, a change that seems essentially to be caused by the spontaneous compensation of the respiratory alkalosis. This compensation is the result of a decrease in the buffering capacity of the blood. The chemoreflex drive is also responsible for this decrease in the buffering capacity, for without this drive hypoxic hyperventilation would not occur nor consequently would the decrease of the bicarbonates take place (cf. p. 15). The chemoreflex drive is thus responsible for the physico-chemical mechanism, by which the center itself becomes more sensitive to a given production of metabolic carbon dioxide.

The changes in the interaction of centrogenic and chemoreflex components during the course of a prolonged exposure to a constant degree of ambient oxygen deficiency at rest as revealed by the present experiments are graphically represented by fig. 15. It seems that certain correlations can be made between the findings of the present work and the respiratory X theory of HENDERSON, if the mysterious agent is considered to be the same as the chemoreflex drive. As has been pointed out above (p. 22) the respiratory X component may be transferred into the actual component in the hypoxic hyperventilation, for which the oxygen lack is responsible. The remaining component may be regarded

as caused by the need for carbon dioxide removal. By extensive studies of the main physico-chemical factors, which are involved in the physiological mechanism of acclimatization to different ambient oxygen pressures, HENDERSON came to the conclusion that the respiratory X component should essentially be proportional to the amount of alkali in use in the blood. As the blood alkali decreases under the influence of prolonged hypoxic hyperventilation, the respiratory X component should accordingly decrease, and the hypoxic hyperventilation should instead increasingly be maintained by the need for carbon dioxide removal. If this change is expressed as a function of time, essentially the same graph is obtained as in fig. 15, although "need for oxygen" should stand for "chemoreflex drive" and "need for carbon dioxide removal" for "centrogenic drive".

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## VI. Summary.

A critical survey is given of notable conceptions of the chemical regulation of respiration during oxygen deficiency at rest. Earlier and recent work is reviewed, and special regard has been paid to the influence of the discovery of the chemoreflexes upon the validity of earlier theories. Correlations have been made between established facts concerning the hemo-respiratory functions involved in the initiation and maintenance of hypoxic hyperventilation.

Mainly as the result of such correlations between physico-chemical and physiological factors the problem of the present work has arisen, viz. the interaction of centrogenic and chemoreflex chemical control of breathing during oxygen deficiency at rest (p. 38).

The interaction of centrogenic and chemoreflex components in the hyperventilation during acute and prolonged exposures to constant degrees of ambient oxygen deficiency has been followed experimentally in dogs under chloralose. The method of study essentially consisted in observing those respiratory reactions which were caused by selective, temporary and repeated cold-blockings of the carotid chemoceptive signals in certain stages of hypoxic hyperventilation. The vagi were cut, and the cold-blockings thus temporarily eliminated all known chemoreflex influence upon the center. The respiratory reactions were judged by the aid of simultaneous, direct and continuous recordings of the arterial pH and oxygen saturation, and yielded data and conclusions as follows.

*Acute hypoxic hyperventilation:* In the stage of hypoxic hyperventilation, that is characterized by increasing respiratory alkalosis in the arterial blood, the self-engendered activity of the chemosensitive cells of the center — free from the chemoreflex drive — decreases in close relationship to the increasing arterial alkalosis. This decrease of the centrogenic support in the hypoxic hyperventilation is essentially due to decreasing stimulation of the chemosensitive cells of the center by decreasing arterial

acidity. Evidence seems to warrant the conclusion that the intracellular reaction of the center does not differ significantly from that of the arterial blood during acute tolerated oxygen deficiency. The cellular acidity theory must be refuted if taken in the sense that acute oxygen deficiency should induce increased intracellular acidity within the cells of the center. However, it may retain its validity if modified only to postulate that the center responds to changes in its acidity (pp. 60—67).

The chemoreflexes are entirely responsible for the increase in the volume of breathing that is initiated under the influence of acute ambient oxygen deficiency. The chemoreflex drive gradually increases during the development of the respiratory alkalosis, thereby compensating for the gradual decrease in the centrogenic support. In maximum respiratory alkalosis from acute ambient oxygen deficiency, the chemoreflexes may be responsible for the entire ventilation, the chemosensitive cells of the center itself being apneic from missing stimulation. Evidence is presented that the increase of the chemoreflex drive during the development of the respiratory alkalosis is not only determined by the hypoxemic stimulus to the chemoreceptors, but is actually potentiated by the alkaline change per se (pp. 67—73).

*Prolonged hypoxic hyperventilation:* Whereas the chemoreflexes gradually gain the upper hand of the center during the development of respiratory alkalosis, the center again becomes increasingly important when, under the same degree of ambient oxygen deficiency, the respiratory alkalosis gradually and spontaneously becomes compensated. The unbalance between the centrogenic and chemoreflex components thus attains its maximum at the maximum of the respiratory alkalosis. The pace-making action of the chemoreflexes decreases again approximately to its eupneic importance as the compensation of the respiratory alkalosis becomes complete (acclimatization). In this stage of hypoxic hyperventilation the centrogenic breathing is super-normal. This fact seems to be consistent with the conception that the chemosensitive cells of the center react to changes in acidity. The decrease of the chemoreflex drive may possibly be due to a suppressing action of the increased arterial  $\text{cH}$  and/or to adaptation of the chemoreceptors to the hypoxemic stimulus (pp. 73—82).

*Other conclusions from the present experiments:*

Temporary cold-blocking of the chemo- and pressosensory impulses in the sinus nerve under eupnea and after sectioning of the

vagi may produce an increase of the pulmonary ventilation, obviously from the withdrawal of tonic inhibition from the pressoreceptors (p. 43 and fig. 7 A, cf. HEYMANS & BOUCKAERT 1930).

The importance of the chemoreflex drive for the maintenance of the cupneic arterial oxygen saturation under general anaesthesia is demonstrated (p. 55 and fig. 9 A, cf. EULER, LILJESTRAND & ZOTTERMAN 1939).

The hyperventilation set up by decreased ambient oxygen pressure may under certain circumstances be suppressed by inhalation of carbon dioxide (p. 70 and fig. 13).

A sudden increase in the oxygen supply during hypoxic hyperventilation and respiratory alkalosis may — at least under general anaesthesia — be dangerous to the body from missing stimulation of the center and chemoreceptors and from blocking of the chemoreflex drive by the increasing arterial  $cH$  (p. 76 and fig. 14 B, cf. GESELL, LAPIDES & LEVIN 1940).

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*From the Clinic for Gynecology and Obstetrics of the  
Royal Caroline Institute, Stockholm*  
Chief: PROFESSOR AXEL WESTMAN

# METABOLISM AND STERILITY OF HUMAN SPERMATOOZOA

BY

ANDERS WESTGREN

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STOCKHOLM 1946



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## PREFACE.

The following work represents investigations carried out during 1944, 1945, and 1946 at the Clinic for Gynecology and Obstetrics of the Royal Caroline Institute in Stockholm.

Professor AXEL WESTMAN, the head of the clinic, suggested the original plan for this investigation. I wish to express my warmest thanks for the great interest he has shown in my work, which he has furthered with valuable, critical discussions of its clinical details and by generously placing the resources of his clinic at my disposal.

I have a great debt of gratitude to Docent ÅKE ÖRSTRÖM, who has followed the development of this work with unfailing interest. He has always been ready to give me the benefit of his great experience, and my work owes a great deal to several stimulating discussions with him.

Most of the investigations have been carried out at the Department for Metabolic Research of Wenner-Gren's Institute. I wish to tender my sincere thanks to the head of that department, Docent JAKOB MÖLLERSTRÖM, who has allowed me to work in his well equipped laboratories and to utilize the valuable resources of his department. My friend and fellow-worker in that laboratory Dr ULF BORELL, I thank heartily for his valuable advice and perpetual interest in my research.

My investigation has been carried out on material which has simultaneously been examined at the special laboratory for sperm analysis of the Caroline Institute. I would express my thanks to the chief of that laboratory, Dr ERIC NORDLANDER, for the information regarding the clinical background of the samples and the results of the routine analysis, and to Miss BRITA HÖGBERG for making these determinations. I would also express to Mr BERTIL MATÉRN M. A. my great appreciation for his valuable help in the statistical working-up of the material. The much-needed improvements in the linguistic get-up of this thesis have been provided by Dr DUDLEY CHEESMAN.



I owe my greatest debt of gratitude to my wife Mrs BIRGITTA WESTGREN for her comradeship and interested assistance in my work.

The investigations have been facilitated by financial assistance, from the "Therése and Johan Anderson Foundation", the special fund of the Medical Prize Group of the Nobel Foundation, and a scholarship from the Swedish Government for the furthering of medical studies.

Stockholm, February 1946.

*Anders Westgren.*

## Introduction.

Human sterility is due to the breakdown of the physiological intercourse between two individuals, of whom one may be partially or totally responsible for the failure. For obvious reasons the biological method used in the breeding of domestic animals for the elucidation of problems of sterility, cannot be used here. The physician is therefore faced with the problem of elucidating the causes of sterility only by careful examination of both partners of the sterile couple. During the last decades considerable progress has been made in clinical methods for the examination of sterility, but there are still many cases, perhaps the majority, where no certain diagnosis can be made.

In the examination of the male partner we have one advantage which is not available in the case of the female: we can study the sex cells directly. The ideal method for the diagnosis of male sterility would naturally differentiate the samples of sperm into two groups, one from the fertile and the other from the sterile men. Unfortunately, no method is yet known, that makes this possible.

Our knowledge concerning the morphological and physiological properties of the spermatozoa is comparatively recent and many important details are still lacking. In the diagnosis of sterility we are only interested in the differences observed in spermatozoa in cases of fertility and sterility. This problem has been studied morphologically for at least twenty years and many valuable results have been obtained. Especially after the work of MOENCH (1930, 1936, and 1940), which seemed to give an exact method for the appraising of the sperm sample by morphological methods, the problem seemed to be solved. Unfortunately this inference was premature, as has been pointed out by, among others, WESTMAN (1944). Other investigations, especially those of SCHULTZE (1937, 1938), have not given the same favourable results, and at present the question whether a morphological analysis is sufficient to give an exact diagnosis of the state of fertility of the donor of the sample, cannot be answered in the affirmative.

The study of spermatozoa by biochemical methods for the elucidation of cases of sterility is much more recent. It has hitherto provided only conflicting results in the question, whether an actual difference can be established between the metabolisms of normal and pathological sperm samples.

The object of my work has been to study the metabolism of spermatozoa by physiological methods and thus to find a suitable method for the differentiation between normal and pathological sperm samples.

## CHAPTER I.

### Review of Former Investigations.

#### The Physico-chemical Properties of Human Semen.

Biochemical analyses of the human semen have been performed by several workers, e. g. by McCARTHY, STEPITA, JOHNSON, and KILLIAN (1928), HUGGINS and JOHNSON (1933), GOLDBLATT (1935), and HUGGINS, SCOTT, and HEINEN (1942). The figures of the last workers have been obtained from 56 normal men, and the properties of semen, prostatic fluid and the fluid from the *vesiculae seminales* have been examined separately. Some of these data will be given here:

	Semen	Prostata	Ves. seminal.
pH .....	7.19	6.45	7.29
Total CO <sub>2</sub> in micromol. ....	24	4.2	
Acid phosphate in micromol. ....	23.8	1.09	14.7
Glucose in mg per cent.....	295	16.4	390
Lactic acid in mg per cent.....	95 <sup>1</sup>		

The proteins of seminal plasma mostly consist of proteoses, the concentration of globulin is only 1.2 per cent. With electrophoretic methods ROSS, MOORE, and MILLER (1942) have found less than 0.02 per cent albumin. The latter authors and SIKORSKI (1943) have studied the electrophoretic patterns of seminal plasma from some abnormal samples of human semen and have found no difference from the normal.

On ejaculation the spermatozoa become immersed in a solution with high buffering capacity owing to its high concentration of phosphates, proteins, and bicarbonate and with a high concentration of glucose.

The enzyme hyaluronidase has been the object of much study in recent years. When hyaluronic acid, a highly viscous substance, is

<sup>1</sup> BERNSTEIN and SLOVOHOTOV (1933) have found 40—50 mg per cent in human semen.

decomposed by this enzyme the viscosity is lowered and reducing substance is given off. The presence of hyaluronidase in the mammalian testis and sperm has been demonstrated and experiments by ELISABETH FEKETE and DURAN-REYNALS (1943) and ROWLANDS (1944) have shown its importance in the process of fertilization. The former authors studied the solvent action of testis extract on the cells surrounding the ovum of the rat. The latter demonstrated that if hyaluronidase was added to the spermatozoa to be inseminated, the amount of sperm cells could be reduced to numbers which otherwise would have had no fertilizing effect.

In 1930 KURZROCK and LIEB demonstrated that if semen was allowed to act on strips taken from the muscular wall of the human uterus, the strips reacted by contraction or relaxation. The effect was very variable. VON EULER (1934, 1934, and 1936) and GOLDBLATT (1935) have studied this question separately, finding substances resembling adrenaline in human semen. ZELLER and JOEL (1941) have demonstrated the presence in seminal plasma of diamino-oxidase, monoamino-oxidase, and choline esterase. HUGGINS and NEAL (1942) have found proteolytic enzymes in semen. A fibrinolysin and a substance inactivating fibrinogen are secreted from the prostate gland. The physiological functions of all these substances are unknown.

GOLDBLATT (1935) has found in human semen an enzyme similar to the starch-splitting diastase found by KARASSIK (1927) in dog's semen. Its activity in the pH range of 7.0—7.5 most often met with in human sperm samples was not measured.

MACLEOD and HOTCHKISS (1942) determined the concentration of spermatozoa, lactic acid, glucose, and reducing substance in samples of semen which had been divided into two portions. In the first 40 per cent of each sample 75 per cent of the spermatozoa were found; the concentration of lactic acid was higher and that of glucose lower than in the other part of the sample. Samples without spermatozoa, from cases of azoospermia, had a high concentration of glucose and little lactic acid (about 45 mg per cent).

SCHERSTÉN (1936) has reported that human semen contains large amounts of citric acid, mostly derived from the prostate gland.

The effect of the variation of pH in combination with various organic and inorganic ions has been studied by several workers, e. g.

SHEDLOVSKY, BELCHER, and LEVENSTEIN (1942) and BROWN (1943). The spermatozoa were most motile at pH 7 to 9 (MUSCHAT), and if they had been inactivated by an unsuitable hydrogen ion concentration they could be restored to normal activity by bringing this factor to a more suitable value. The addition of sulphapyridine and sulphanilamide in clinically therapeutic concentrations had no effect on the longevity of the spermatozoa. SHEDLOVSKY and coworkers studied the effect of various acids and alkalis, finding that only acetic acid, monochloroacetic acid and ammonia had a spermicidal effect that exceeded that caused by the change of pH.

Comparatively little has been done concerning the analysis of human spermatozoa in contrast to other mammalian spermatozoa. ZITTLE and O'DELL (1941) in a few cases have found conditions in human spermatozoa similar to those of other mammals. In these cells large chemical differences were found in the various parts. The greater part of the lipid content was found in the tails, the heads having the highest phosphorus content. A large amount of sulphur was found mostly in the form of cystine or cysteine. The presence of glycogen in human spermatozoa has not been demonstrated.

### Motility of Human Spermatozoa.

The question of the rate at which the spermatozoa can travel through the genital tract has long been in the centre of clinical interest. TSUKAMOTO (1932) and before him ADOLPHI (1905) have studied the velocity of spermatozoa under the microscope and found it to be about 1.4 mm/minute. The rate *in vivo* probably exceeds this.

OHLIN (1935) has studied the question whether the epithelium of the tubes can maintain motility of the spermatozoa in any measurable degree, but has found no such effect.

The diagnostic value of determinations of the motilities of the spermatozoa in cases of sterility has been very differently estimated. The only author who considers the degree of motility to be of great importance for the appraisal of the sperm sample is HUFFMAN (1941). He also characteristically points out how easily the spermatozoa are damaged and lose their motility if not carefully treated before the examination. HOTCHKISS (1941), WEISMAN (1941, page 72), and HAMMEN (1944) all attribute some importance to the degree of motility, but point out that otherwise quite "normal" sperm samples

sometimes have low motility. The figure for the per centage of immotile spermatozoa, that should be considered as pathological varies for different workers. WEISMAN gives the figure of 20 per cent, but HAMMEN has found more than 40 per cent of immotile cells in many otherwise normal samples. MOENCH (1939), JOEL (1941), and VARNEK (1944) all state that "motility is not a criterion of fertility".

In conjunction with these problems another method for the analysis of the properties of the sperm sample should be mentioned. In this method the sample is stored at standard temperature, usually protected from evaporation and is examined at regular intervals in order to determine how long the spermatozoa retain motility under these circumstances. The conditions under which the sample is stored vary for different workers; they are described for example, by WEISMAN (1941, p. 73). This author has also studied the correlation between the temperature in which the sample is stored and the longevity, viz. the ability to retain motility. Normal spermatozoa usually retain motility at room temperature for 24 hours, at 34—35° C. for 18 hours, and at 37.5° C. for 10 to 12 hours. WEISMAN (1941, page 74) remarks that the sample must be carefully treated before the examination if the method is to give accurate results.

### Metabolism of Spermatozoa.

As research work on the metabolic processes of spermatozoa has primarily been directed on animal sperm, this work will now be reviewed before the corresponding work on human spermatozoa. Similarly, in the next chapter the results acquired by using the metabolic properties of animal spermatozoa as standards of fertility, will be dealt with before the corresponding work on human spermatozoa.

The first demonstration of the respiration of spermatozoa was made by GRAY (1928). Using the BARCROFT-HALDANE manometer he further showed how the respiration increased on dilution of the suspension of spermatozoa taken from the sea-urchin. Both WINDSTOSSER (1935) and WINBERG (1939) have found similar reactions in suspensions of mammalian and cock's spermatozoa respectively. Measuring the value of  $ZO_2$  (the respiration in  $\mu$  l./hour and  $10^6$  cells) of the spermatozoa from different mammals, LARDY and PHILLIPS (1943) have obtained very different figures (bull 21, cock 7, rabbit 11, and ram 22).

The source of energy for the motility of the spermatozoa has been the object of much work since 1931. In that year IVANOV called attention to the fact that neither the addition of cyanide nor the complete evacuation of oxygen could stop the movements of the spermatozoa which showed that the energy of motion was not obtained through respiration.

Using the WARBURG technique, REDENZ (1933) demonstrated that the spermatozoa taken from quinea-pig and rabbit also had full motility in the absence of oxygen and he could measure the formation of acid metabolites. He came to the conclusion that glycolysis was probably the source of energy. IVANOV (1935) then demonstrated that motility proceeded even if both these reactions were stopped. He had blocked glycolysis with moniodoacetic acid in the absence of oxygen and it became evident that the cells had another source of energy. LARDY and PHILLIPS (1941 and 1944) have studied this problem on bull sperm and found evidence of an intracellular reserve of energy in the form of phospholipids (see also p. 16).

The degradation of glucose and formation of lactic acid in bull and human semen have been studied by BERNSTEIN and SLOVOHOTOV (1933, cf. ANDERSON p. 78). Unfortunately the papers describing this work have not been accessible to the author except in abstract, and important details are therefore lacking in this recapitulation. The lactic acid was determined according to the methods of FRIEDEMANN, COTONIO, and SHAFFER and of MENDEL-GOLDSCHNEIDER. The concentration of the glucose decreased when the sample was stored and the motility of the spermatozoa was highest when the curve of glucose splitting was steepest. Occasionally a slow splitting of glucose occurred in samples where no motile cells were present. The formation of lactic acid varied with the density of the suspension, the intervals between the matings and the time between collection of the ejaculates and examination. The end of motility for the spermatozoa could not be explained by the glycolysis figures. Usually a large part of the glucose was still present in the solution and the concentration of lactic acid at the end was very variable. These results must be seen in conjunction with the results of KARASSIK (1927), who has found a diastatic enzyme in dog's semen and pointed out its importance for the glycolysis of the spermatozoa (for further discussion see also pp. 10 and 56).

MANN (1915) has studied the anaerobic metabolism of ram sperm.



He was especially interested in the phosphorus metabolism of the cells and found several of the important metabolites that are typical for the breakdown of glucose with the help of adenosine triphosphoric acid.

The presence of several other important metabolic factors have been demonstrated by different workers. LARDY and PHILLIPS (1941) have found succinic acid dehydrogenase, riboflavine, and an active alkaline phosphatase in the semen of bull. WINBERG (1941) found a comparatively high concentration of cozymase in the spermatozoa of the cock. When respiration, as measured in the WARBURG apparatus, had stopped, the concentration of cozymase had decreased to only 40 per cent of the original figure. Spectroscopic determinations by MANN (1945) revealed the presence of cytochromes *a*, *b*, and *c* in ram sperm.

Determinations of the metabolism of human spermatozoa have been made only since 1928. In that year MCCARTHY, STEPITA, JOHNSON, and KILLIAN demonstrated how the concentration of reducing substance decreased when a sperm sample was stored, and found a formation of lactic acid. KILLIAN (1933) confirmed the formation of lactic acid and showed how the pH changed in a specimen which was not protected from evaporation. In these specimens the pH rose steeply at first because of the loss of CO<sub>2</sub> to the air; it then gradually decreased. KILLIAN claimed that the decrease of pH resulted from the formation of lactic acid by the spermatozoa, but this can be doubted when the relatively small amounts of lactic acid formed by suspensions of human spermatozoa are compared with the high buffering capacity of the sperm plasma.

Different opinions have been held concerning the problem whether the spermatozoa form a proportionate amount of lactic acid from the glucose they consume. If one molecule of glucose were split and totally converted into lactic acid, two molecules of lactic acid would be formed. The quotient between the amounts in micromoles of lactic acid formed and glucose consumed by the spermatozoa would be 2.

MCCARTHY et al. (1928) and GOLDBLATT (1935) have found that much more glucose disappears than would have been consistent with the complete conversion of glucose to lactic acid. KILLIAN (1933), on the other hand, has found a proportionate formation of lactic acid. All these determinations were made in much the same manner. The sperm samples were incubated for a certain time, often as long

as 24 hours, and the concentrations of glucose and lactic acid were determined before and after the incubation. It has been pointed out by MACLEOD (1943) that serious errors due to the growth of bacteria were probably introduced into these determinations, and the problem is therefore still unsolved.

SHETTLES (1939) investigated these problems and used the WARBURG technique for the determination of respiration and glycolysis (formation of acid metabolites). He found the respiration of the sample was directly proportional to the number of spermatozoa present. ROSS, MILLER, and KURZROCK (1941) studied the same problem using the methods of DICKENS and SIMER for measuring the respiration and glycolysis. These authors separated the spermatozoa from the plasma by centrifuging and resuspended them in a solution of RINGER-bicarbonate in equilibrium with a gas mixture of 1.2 per cent  $\text{CO}_2$  and 98.8 per cent oxygen. In contrast to the results obtained by SHETTLES, they found the respiration of the spermatozoa negligible. The plasma proved to have a much greater respiration than the cells. The degree of motility and the density of the suspension were probably of significance for the metabolism. There were great variations between the values for the metabolism of sperm specimens from the same individual at different times.

Since 1939, MACLEOD has published several papers on the metabolism of human sperm cells (1939, 1941, 1942 and 1943). After separating the spermatozoa from the plasma by centrifuging he suspended them in a RINGER solution, usually adding sufficient glucose to make the concentration 200 mg per cent solution. For determinations of respiration he added a 1/15 molar phosphate buffer to pH 7.4, and for glycolysis a 0.03 molar bicarbonate solution. In the first case he used the differential method of DICKENS and SIMER, in the second the direct method of WARBURG. Like the former authors he found the respiration to be insignificant ( $Z_{\text{O}_2} = 1.8$ ). The cells were more liable to a decrease of activity in an oxygen atmosphere than under anaerobic conditions. The formation of acid metabolites (mostly lactic acid) was more active anaerobically ( $Z_L^{\text{O}}$ : 9,  $Z_L^{\text{N}}$ : 11).

As substrates for their metabolism the spermatozoa could employ maltose, mannose, fructose, and glycogen, but saccharose and galactose were unsuitable. A concentration of more than 600 mg per cent glucose was harmful.

A slight contamination with bacteria was usual. The samples had

been obtained from normal men by manipulation or *coitus interruptus*. Three specimens, free from microorganisms, showed exactly the same metabolic behaviour as the "non sterile" specimens. Bacterial growth was manifested by an increase in the formation of acid metabolites, which increased logarithmically. This did not take place until the sample had been stored at body temperature for more than three hours.

The individual acid production remained relatively constant from week to week. Sharp rises and falls occasionally occurred which could not be accounted for on the basis of motility. No significant difference could be found by MACLEOD in the metabolism of samples of differing cell-morphologies.

According to the opinion of MACLEOD there are two systems of metabolism in the human spermatozoon:

1. Glycolysis, with the production of lactic acid from glucose. The sperm cells acquire their motive energy from this. The reaction can be stopped by addition of monoiodoacetic acid. Lack of suitable sugar results in the suspension of all motility after 2 to 4 hours. The movements begin again if such a sugar is added, unless the cells have been exposed to oxygen or been immotile for more than half an hour.

2. A cytochrome system. The author states that the presence of cytochromes *a* and *b* has been proved indirectly. On addition of succinate the respiration increases but motility stops.

A high oxygen tension checks the motility and metabolism of spermatozoa. If haemoglobine or catalase is added, this effect can be avoided. The author suggests the possibility of the formation by the sperm cells, as by certain microorganisms, of hydrogen peroxide, which they are unable to handle with their incomplete enzyme system.

### Metabolism as a Standard of Fertility.

WALTON and EDWARDS (1938), using BARCROFT-DIXON manometers, measured the respiration of sperm samples from bulls. In these experiments they did not separate the sperm from the plasma but only diluted the samples with a phosphate buffer. They demonstrated that the respiration was higher in samples from bulls of good fertility than in those from sterile bulls. COMSTOCK and GREEN (1939) published similar investigations on the respiration and formation of acid metabolites in sperm samples from bull and goat. In these

determinations they did not compare the metabolism with the fertility of the bull but with the longevity of the cells, viz. the ability of the spermatozoa to remain motile for a sufficiently long time. The correlation was 0.87 for the glycolysis and 0.58 for the respiration.

A new method for obtaining criteria of fertility was tried by SÖRENSEN (1942). He used a modified THUNBERG technique, measuring the rate of dehydrogenation by suspending the spermatozoa in a solution of methylene blue. He found a very good correlation between the time of decolorization and the amount of spermatozoa motile in the suspension.

KLEIN and SAROKA (1941) have used a similar method to measure the metabolism of *human* spermatozoa. They determined the rate of dehydrogenation and found this to be a good way of appraising the specimen and the fertility of the donor. If the sample took more than one hour for the decolorization, the donor probably was sterile. A similar method has been tried by HAMMEN (1944), but his results have not been very satisfactory. The time consumed often exceeded one hour and in all such cases it is probable that factors other than the metabolism of the spermatozoa interfered with the reaction.

ROSS, MILLER and KURZROCK (1941) have studied the metabolism of spermatozoa with manometric methods as mentioned in the preceding chapter. If the samples were divided into three groups, 1) fertile, 2) probably fertile and 3) probably sterile, there was no difference between the groups in the rate of formation of acid metabolites but some difference in the rate of respiration. This latter deviation was, however, so small that the results were not quite reliable.

Morphological analysis has been considered the best method of differentiating normal and pathological sperm samples, but MACLEOD has not been able to find any correlation between the metabolic and morphological properties of the spermatozoa.

Finally, I must own that owing to the war and its restrictions on the circulation of scientific literature, it is possible that some important papers on the subject in hand have been too briefly reviewed or possibly overlooked.

## CHAPTER II.

### Plan of the Investigation.

The object of the present work was to find a suitable clinical method for the elucidation of cases of male sterility by the examination of sperm samples. As the methods are intended to be used clinically, they should not be excessively complicated or time-consuming. They should give well-defined and objective measures of the properties of the sample, especially of the density of the suspension of spermatozoa, the degree of motility and the metabolic rate of the cells.

After a review had been made of the methods already used for this purpose or apparently adaptable thereto, the following plan of investigation was drawn up.

For the determination of the density of suspensions of spermatozoa, the method which has been most widely used was that originally worked out by BELDING (1934). The spermatozoa are diluted with a liquid which stops their movements, whereafter they are counted on a counting slide. The determination of the degree of motility of the cells is usually made by observing a certain number of cells in a drop of sperm on a slide under the microscope and counting the motile and non-motile cells. The result can be given as a percentage or in other manners which vary for different workers. A difficulty of this method is the counting of the motile cells if their percentage is high and especially if their speed is great.

Another weakness is the impossibility of obtaining identical conditions for different samples, since the thickness of the sperm layer varies with the viscosity of the sample. These difficulties have been avoided in a method described by CLARE HARVEY (1945) which has been employed in this work (see p. 23).

Use was made of the manometric method first described by WARBURG for measuring the formation of acid metabolites, since this had

been employed by most of the workers in this field, and thus appeared to be fairly well developed (see p. 25). Another advantage of this method is that it readily permits the simultaneous use of other methods for studying the properties of the object under investigation.

In some experiments determinations were performed, according to the "direct" method of WARBURG of the respiration rates of suspensions of spermatozoa. As, however, these seemed very low or even impossible to measure, in agreement with the results of ROSS et al. (1941) and MACLEOD (1941), this method appeared unsuitable for the purpose.

The colorimetric determination of lactic acid according to the method of BARKER and SUMMERSON (1941), (see p. 27), seems not to have been used for the study of the metabolism of human spermatozoa except by MACLEOD and HOTCHKISS (1942). It has a high specificity and it seemed possible that, if it were combined with the WARBURG method, valuable new information about the sperm metabolism could be acquired.

Determinations of the rate of dehydrogenation for similar purposes have given conflicting results and it was therefore preferred not to use this procedure.

As mentioned on page 14, different views have been held, as to whether or not the spermatozoa form a proportionate amount of lactic acid from the glucose they consume. It was hoped to solve this problem by determining the decrease in the glucose concentration during the experiment. For various reasons the HAGEDORN-JENSEN method was adopted for the determining of the concentration of reducing substance (see p. 29). It was well suited to this end, since it was already used for clinical purposes. Several objections could be raised against other possible methods. The determination of glucose by yeast-fermentation would have been too tedious and its accuracy is open to doubt. The employment of oreinol for the determinations was out of the question because of the lack of the necessary reagents.

The morphological properties of the sperm cells were to be analyzed simultaneously and it was of great interest to compare them with the results obtained by the biochemical methods. It was intended to make this comparison when the chemical data had been worked out.

## CHAPTER III.

### Methods Used in the Investigation.

#### General Course of a Determination.

The determinations of the metabolism of sperm samples were performed during the period April to December 1945. There was only one short interval in the work and all the determinations were made by the same persons and in the same manner. The samples from donors of different grades of fertility (see p. 32) were examined in parallel. It was attempted to avoid examining series deriving from only one group (see p. 34) during a given period.

The sample from the donor was obtained either by *coitus interruptus* or manipulation. It was immediately placed in a glass receptacle. Rubber sheaths were never used as receptacles. The glass was kept in an upright position and guarded from sudden changes of temperature, but it was allowed to cool to the temperature of the room in which it was stored. It was delivered at the laboratory of the Caroline Hospital, after which a part was taken for the biochemical investigations and transmitted to Wenner-Gren's Institute. During the winter the samples had to be transported in a thermos flask kept at room temperature.

The sample was centrifuged for five minutes at 4 500 r. p. m. in an angle centrifuge. Owing to the sometimes very high viscosities of the samples, it became necessary to use a centrifugation speed as to sediment the cells of all samples. The supernatant fluid was decanted off and the sediment of spermatozoa was resuspended. The suspensions were made very carefully by gentle suction and blowing in a 1.0 ml pipette. The formation of foam and other signs of blowing air through the suspension were carefully avoided. The fluid used for the suspension was a RINGER-bicarbonate solution (see p. 25). The author has tried another fluid for this purpose, the so called KREBS-RINGER solution (KREBS and HENSELEIT 1932), but this very

readily became turbid and no difference was found between the formation of acid metabolites in this fluid and in the more easily prepared RINGER-bicarbonate solution. The latter has also been recommended by MACLEOD (1941).

The total volume of liquid in each flask for the WARBURG determinations was 3.3 ml. Before these determinations began, however, samples were removed from the vessels for determinations of the amounts of lactic acid (0.5 ml) and reducing substance (1.0 ml). The total amount of liquid therefore had to be correspondingly in excess of 3.3 ml (viz.  $3.3 + 0.5 + 1.0 = 4.8$  ml). This volume was made up of 1.0 ml spermatozoon suspension, 3.5 ml RINGER-bicarbonate, and 0.3 ml 480 mg per cent glucose solution.

Where this was possible, determinations were made in duplicate. In such cases the suspensions had to be diluted to proportionately larger volumes.

The plasma, i. e. the supernatant fluid after the centrifugation, was diluted if necessary with RINGER-bicarbonate to 1.0 ml and was added to a WARBURG flask in the same way as the suspensions. As the sample for the determination of the concentration of reducing substance in the plasma-flask was only 0.5 ml, the initial volume here was only 4.3 ml made up of 1.0 ml plasma, 3 ml RINGER-bicarbonate, and 0.3 ml glucose solution.

The study of the metabolism of the sperm plasma was undertaken because it proved practically impossible to free the suspension entirely from the plasma unless the cells were centrifuged and resuspended so many times that they were damaged in their activity. The values obtained with such samples can be studied in Table 1 and in the graphs of Fig. 1.

The plasma volume which still accompanied the spermatozoa after centrifugation and resuspension was not more than at most 0.2 ml per ml suspension transferred to the Warburg flask (for discussion on this point see p. 56). The study of the three cases which had been centrifuged more than once, led to the conclusion that the centrifugations damaged the activity of the spermatozoa, as evidenced by the decrease in the number of motile cells, and the reduced formations of lactic acid and acid metabolites. The consumption of reducing substance, however, showed an increase after two centrifugations. When the figures for the percentage of motile cells before the first centrifugation are studied, they are seen to be lower after centrifugation.



Table 1.

Influence of centrifugation and resuspension on the activity of the spermatozoa.

Number of sperm-sample	No. of Centrifugations	Total number of spermatozoa in $10^8$	Percentage of motile cells	Number of motile cells in $10^8$	Lactic acid per hour in micromol.	Acid metabolites per hour in micromol.	Reducing substance consumed in micromol. per hour
542	0		75				
	1	0.33	77	0.25	0.73	0.70	0.23
	2	0.33			0.62	0.74	0.32
	3	0.33	0	0	0.42	0.17	0.20
561	0		35				
	1	0.24	23	0.06	0.13	0.13	0.07
	2	0.24			0.03	0.07	0.16
	3	0.24	0	0	0.04	0.04	0.06
620	0		56				
	1	0.06	51	0.03	0.08	0.15	0.03
	2	0.06			0	0.12	0.13
	3	0.06	0	0	0	0.07	0

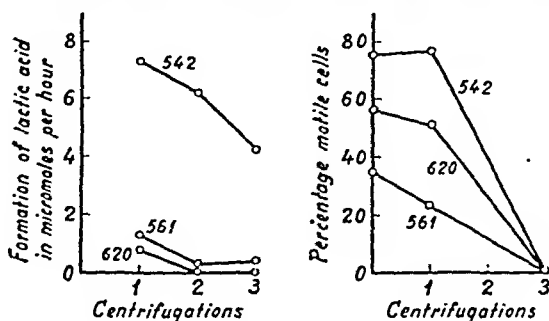


Fig. 1. Effect of centrifugations on metabolism of spermatozoa.

gation in two cases of the three, which makes it likely that the samples are damaged even by the first centrifugation (for further discussion on this point, see p. 47).

## The Determination of the Density of the Suspension of Spermatozoa and the Percentage of Motile Cells According to CLARE HARVEY.

A sample of the suspension of spermatozoa was taken from the WARBURG flask in a 0.5 ml pipette and small drops were placed alternately on the squared surfaces of the two counting slides, each of which had two such surfaces. One of the slides was placed for 10 seconds under the lid of the box in which the slides are sold. Under this lid had been fixed a piece of blotting paper moistened with a drop of a 2 per cent solution of osmic acid. The cover-slips were then fixed carefully on both slides and the formation of NEWTON's rings at the surface between the counting slides and the cover-slip could be established. The immotile spermatozoa were now allowed to settle to the bottom for five minutes after which the counting was made. At first the non-motile cells on the untreated slide were counted; subsequently the total number of immotile, osmiumtreated cells was determined. In each determination more than 100 cells were usually counted, half of them from each "side" of the slide. From these figures the concentration of the spermatozoon suspension and the percentage of motile cells could be calculated. The concentration of spermatozoa was estimated in the same way as for blood-corpuscles in blood. The percentage of motile cells was calculated from the figure obtained by subtraction of non-motile cells from the total number of cells.

No attempt was made to describe the velocity and other properties of the spermatozoon movements as no means was available for recording these objectively.

In a few samples of high density it was necessary to dilute the suspensions to make the counting of the cells possible. The dilution was made with an equal volume of RINGER-bicarbonate (see p. 25) at 37° C.

Unfortunately the microscope was not fitted with a heated stage, so that motilities had to be examined at room temperature. As a precaution against a change in the motility, the time between the taking of the sample from the WARBURG bath and the examination was reduced as much as possible, usually not exceeding ten minutes. The percentages of motile cells were estimated before the WARBURG determinations in the first samples, but the figures thus obtained were lower than those found after the end of the determination. As evi-

Table 2.

Influence of the conditions in the WARBURG determination on the percentage of motile cells in the suspension.

Number of sample	Percentage of motile cells			
	before WARBURG determination	after one half hour in the WARBURG flask	after one hour	after three hours
452	10	20	25	24
463	60	82	83	80
473	25	54	50	51
474	68	80	75	81

denced by the figures in Table 2, the percentage of motile cells rose soon after the temperature of the sample had been increased. The initial figure therefore did not represent the number of motile cells actually present in the flask during the determination. These determinations were therefore abandoned, and counts were henceforth made only at the end of the experiment.

As the method of CLARE HARVEY is new, I have tried to ascertain its possible weaknesses. In the usual procedure the drops of sperm are placed beside the cover-slips *in situ* and the liquid is sucked in under the slip by the capillary force. In the new method, however, the drops are placed on the surface of the counting slide and the slips later placed on top of the drops. Repeated determinations of the density of a suspension which was alternately sucked in under the slip and placed on the bare counting slide, gave the same values. It is to be remembered that in the present work no determinations have been made on highly viscous suspensions where the viscosity might interfere with the results.

Repeated control determinations of the densities of suspensions on both slides gave practically identical results (error of mean  $\pm 1$  per cent). In the first fourteen determinations control counts of the total numbers of motile and non-motile cells were made and the sum was compared with the total number of cells in the osmium-treated chamber. With only two exceptions the sums were lower than the total number, the mean difference being 6 per cent. The explanation of this seems to be the difficulty in counting the motile cells correctly.

As a precaution against the possible retention of osmic acid on the osmium-treated slide, this was never used for the motile cells.

The variation between the numbers of spermatozoa found in different drops from the same suspension has been determined statistically. In 40 cases the percentage error in the mean was  $\pm 1\%$ , the range of variation extending from 84 to 110. The figure for the percentage of motile cells is obtained by subtraction of one figure for the concentration of a group of cells from another, so that the mean error in the motility is  $\pm 1.5\%$ .

### The WARBURG Method for the Measurement of the Formation of Acid Metabolites.

The construction of the WARBURG apparatus and the theoretical basis of the method will not be dealt with here, but reference should be made to the excellent works already available (DIXON 1934, DICKENS 1940, and UMBREIT, BURRIS, and STAUFFER 1945). The principle of the method is that the acid metabolites formed by the spermatozoa change the equilibrium of a buffering system of bicarbonate and gaseous  $\text{CO}_2$ . More  $\text{CO}_2$  is formed and the manometer reacts and registers increased pressure.

The suspension medium used in this work was the RINGER-bicarbonate solution recommended by MACLEOD (1941). It consists of 16 parts of 2.97 per cent  $\text{NaCl}$ , 10 parts of 1.3 per cent  $\text{Na}_2\text{CO}_3$ , 1 part of 1.1 per cent  $\text{KCl}$ , 1 part of 0.11 M-  $\text{CaCl}_2$  and 32 parts of distilled water. The solution of  $\text{Na}_2\text{CO}_3$  had always been treated with  $\text{CO}_2$  for at least half an hour immediately before use. The pH of the RINGER-bicarbonate was checked and remained at 7.3 with small variations.

In every vessel 0.3 ml of a 480 mg per cent solution of glucose was added to give the desired glucose concentration of about 30 mg per cent. The volumes of the flasks varied between 18 and 21 ml. The volume of the liquid was always 3.3 ml. Throughout the investigation a gas mixture of 5 per cent  $\text{CO}_2$  and 95 per cent nitrogen was used. The perfusion time before the closing of the vessels was 45 seconds. The gas mixture was chosen according to the results of MACLEOD (1941 and 1942). At least twenty minutes were allowed to pass before any readings were taken of the pressure in the flasks; in this time the temperatures had time to level out. Regular readings were then made every half hour and the determinations lasted for three hours.

This time was chosen as the result of a study of the metabolic rates of several samples run for longer times (see Fig. 2).

The shortest time needed by any of these samples to show evidence of a logarithmically increasing formation of acid, a sign of the activity of microorganisms, was five hours. The presence of bacteria in sperm suspensions is usually not manifested until between the third and sixth hour, according to ANDERSON (1943).

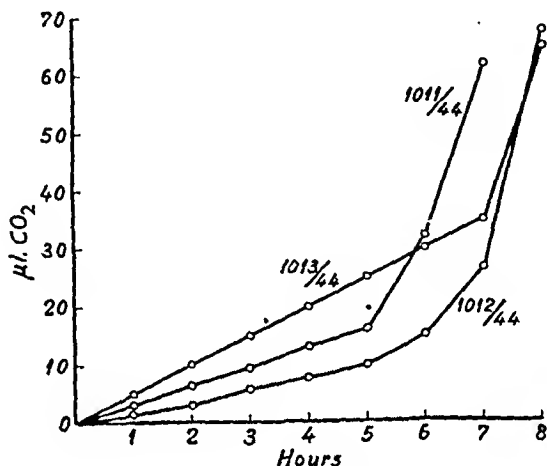


Fig. 2. Metabolic rate of spermatozoa.

With the help of a thermoregulator, the temperature of the bath was maintained practically constant at  $36.6^{\circ} \pm 0.2^{\circ}$  C. The manometers were rocked at a constant rate of about 120 oscillations per minute. After three hours the vessels were removed from the bath, samples were taken and the pH was determined, on some occasions with a BECKMAN potentiometer but usually with Lyphan paper. The pH was always the same as before the determination.

After correction by the vessel constant, the figures for the formation of  $\text{CO}_2$  in every half-hour interval could be calculated. These figures never showed any increase towards the end of the determination and only small variations for each half hour (see Fig. 19). The mean value for the amount of  $\text{CO}_2$  liberated in one hour was then divided by the gas equivalent 22.4, thus giving the formation of acid metabolites in micromoles per hour.

An attempt was made to estimate the range of variation of the values obtained with this method; to this end the twelve cases with

determinations in duplicate were treated statistically. Expressed in percentages the range extended from 94 to 117 and the error in the mean was  $\pm 2.3$ .

### The Colorimetric Determination of Lactic Acid According to BARKER and SUMMERSON.

This method may be briefly described as follows: Lactic acid in concentrated sulphuric acid is converted into acetaldehyde. The latter reacts with p-hydroxydiphenyl in the presence of an optimum concentration of copper sulphate with the formation of a violet-blue colour. This can be measured in an electrophotometer.

As suggested by BARKER and SUMMERSON (1941), standards with known concentrations of lactic acid were always measured at the same time as the samples. The standard solution was made each day by diluting a 0.1 molar solution of lactic acid to give the desired concentration of 10  $\mu\text{g}$  per ml. The experimental procedure was as follows:

A series of centrifuge tubes were prepared before the determinations, each containing 1.0 ml of a 20 per cent  $\text{CuSO}_4$  solution. The standards, four in number, contained in addition 2 to 4 ml of the standard solution. Water was added to make the total volume 10 ml. The volume taken from suspensions or plasma samples was always 0.5 ml. When the samples had been taken, 1.0 ml of a suspension of 10 per cent  $\text{Ca}(\text{OH})_2$  was added and the tube shaken carefully. In the original method 1.0 g of powdered  $\text{Ca}(\text{OH})_2$  is used, but owing to the war, it was difficult to obtain this reagent chemically pure in sufficient amounts and it was necessary to modify the method in the following manner.

Some control determinations made it evident that a special de-proteinizing process in addition to the precipitation with copper and calcium was unnecessary (see Table 3).

The tubes with the suspension were allowed to stand for at least half an hour with occasional shaking. After centrifuging 1.0 ml of the clear, supernatant fluid was carefully pipetted off without disturbing the sediment and the pipette was wiped clean from the film forming on the surface of the liquid. The sample was transferred to a THUNBERG tube and 0.5 ml of a 4 per cent solution of  $\text{CuSO}_4$  was added. According to BARKER and SUMMERSON wide test-tubes

Table 3.

Deproteinizing effect of the precipitation with copper and calcium.

Sample	Amount of lactic acid in micromoles	
	in untreated sample	in the sample deproteinized with 10 per cent trichloroacetic acid
1	0.30	0.32
2	0.09	0.08
3	0.10	0.11
4	0.12	0.10

are preferable, but in my experience closed tubes of the THUNBERG type are better. They can be shaken more effectually and there is no risk of spraying sulphuric acid.

Exactly 6.0 ml of sulphuric acid is added. The sample is not allowed to boil during the addition. This can be avoided if the tube is shaken vigorously. The tube is then heated for five minutes in a boiling water bath. After cooling to below 18° C., 0.1 ml of 1.5 per cent p-hydroxydiphenyl in a 2 per cent NaOH solution is added. Appropriate amounts of the reagent had been weighed and dissolved in the alkali, but the solution was rather unstable and crystallized easily, so that it had to be handled carefully. Only small amounts of the solution were prepared and they were discarded at the first sign of increased turbidity.

The diphenyl was precipitated and the tube had to be shaken carefully in order to distribute the precipitate evenly. The tube was then placed in a water bath at 30° C. for at least half an hour. It is good practice to give the tube an occasional shake during this time.

The tube is then heated for 90 seconds in a boiling water-bath, the precipitate is dissolved and the liquid becomes clear. The tube is cooled to room temperature and the colour-intensity is determined in an electrophotometer with a filter of peak-transmission 5 600 Å. The concentration of lactic acid is very readily deduced from the extinctions of the standards of the samples, as the extinction curve in this concentration range follows the law of LAMBERT-BEER. The total amount of lactic acid in the WARBURG flask expressed in micromoles is calculated from the value of the sample.

There are some very important sources of error in this method. The first and most important of these has already been mentioned.

It is necessary that the reagents used in the process are pure. According to the very limited experience of the present author the most common impurities occur in the  $\text{Ca}(\text{OH})_2$  and sulphuric acid. A common error is caused by contamination from the person who is handling the tubes, pipetting the fluids and so on. Chemical "sterility" must be rigidly maintained. Another mistake is failure to cool the samples sufficiently. The results of this can be easily observed by a worker accustomed to the normal colour reaction. Under usual conditions the colour of the solution, when it has been placed in the water-bath at  $30^\circ \text{C}$ ., is purely blue and some precipitate still remains undissolved. If the temperature has been too high the colour is violet and the fluid clear. One of the substances interfering with such determinations is acetaldehyde. A modified diffusion technique for the determination of acetaldehyde (ÖRSTRÖM 1945) was used to confirm that no acetaldehyde was present in the sperm sample and that none was formed by either the spermatozoa or their plasma.

During the whole course of the work an automatic pipette was used for  $\text{H}_2\text{SO}_4$ , and LINDERSTRÖM-LANG pipettes (LINDERSTRÖM-LANG and HOLTER 1941) for the p-hydroxidiphenyl and 4 per cent  $\text{CuSO}_4$  solutions.

The total number of determinations made in duplicate by this method was 11. If the results were treated statistically and the variation expressed as a percentage, the range of variation extended from 92 to 111 and the error in the mean was  $\pm 2$ .

### The Determination of Reducing Substance According to HAGEDORN-JENSEN.

A modification of this method described by F. RAFFAPORT (1932) was adopted. The principle of this is that the reduction of  $\text{K}_3\text{Fe}(\text{CN})_6$  takes place in the presence of a phosphate buffer, which makes the determinations, especially of high concentrations of reducing substance, more reliable.

It very soon became evident that the concentration of glucose used by other workers, and also by the present author in his preliminary work, was too high to permit sufficient accuracy in the determinations. The quotient between the amount of glucose consumed by the spermatozoa and the total amount of glucose in the solution was so low that the determinations became very inaccurate.



Table 4.

Effect of reducing the concentration of glucose in the suspension of spermatozoa.

Number of sample	200 mg per cent		30 mg per cent	
	Acid metabolites in micromoles per hour	Percentage of motile cells	Acid metabolites in micromoles per hour	Percentage of motile cells
409	0.23	65	0.20	70
418	0.12	65	0.10	60
420	0.10	50	0.12	54
426	0.32	85	0.35	90

This difficulty was evaded by decreasing the total amount of glucose in the solution. In this way the value of the quotient was increased and the volume of the samples taken could be augmented. As evidenced by the figures in Table 4, there was no evidence of any change in the metabolism of the spermatozoa. For this reason a 30 mg per cent solution of glucose could be employed instead of the former 200 mg per cent. After the determinations the concentration of reducing substance was never so low that all glucose could be considered to have been consumed by the spermatozoa. In the sample with the highest consumption of reducing substance, viz. 0.93 micromoles per hour, the consumption amounted to half the glucose added to the solution. According to MACLEOD (cf. ANDERSON 1943, p. 78) the effective concentration of utilizable sugar lies between 20 and 200 mg per cent.

The concentration expressed in mg per cent of glucose could be calculated from tables in the paper already referred to. These figures were recalculated to express the concentration in micromoles in the total contents of the WARBURG flask.

Sixteen determinations in duplicate had been made with this method. If they were treated statistically and one figure was expressed as a percentage of the other, a very wide range of variation was found. Ten of the determinations gave a value of more than 0.15 micromoles. In these figures the range of variation extended from 61 to 126 and the mean error was  $\pm 7$ . The six other determinations showed values of less than 0.15 micromoles and were therefore more uncertain. The range of variation was 46 to 200 and

the error in the mean was 22. The source of the inaccuracy in the determinations must have been the presence of relatively large amounts of reducing substance of a nature different from glucose.

### Statistical Methods.

A statistical working up of the material is especially valuable, when the biological variation is considerable as in the present material.

The standard deviation was calculated from the formula:

$$\delta = \sqrt{\frac{\sum a^2}{n-1}}$$

where  $a$  = the deviations of the individual values, and  $n$  = the number of determinations made.

In some cases it has been important to compare two mean values, and here "STUDENT'S"  $t$ -test, as described by FISHER (1941) has been employed. The value of  $t$  observed in these calculations was compared with that in the tables of FISHER's work, and the difference obtained was indicated as significant and statistically verified when  $P$  was found  $\leq 0.01$ , and as probable for a value  $\leq 0.05$ .

The correlation between different series of observations has also been measured and similar rules for measuring the strength of the correlation have been observed. The correlation has been considered as strong when the value of  $P$  in the tables of FISHER's work was  $\leq 0.01$  and as probable for a value  $\leq 0.05$ . The relations between the different entities of both series were expressed in regression equations ( $y = a_0 + a_1 x$ ).

## CHAPTER IV.

### Material.

The sperm samples in this work can be subdivided into two groups. The first of these came from voluntary donors of good fertility, who had their sperm samples examined for scientific purposes; the second came from men who sought medical aid for marital sterility and were advised by various physicians to have their sperm examined at the special laboratory for this purpose at the Caroline Hospital.

The first group consisted of 27 samples from the same number of donors. Great difficulty was found in obtaining even this relatively small number of samples; it was impossible to acquire samples more than once from any donor. It was thus impracticable to make control determinations of the metabolic rate of a given sperm at different times.

The 27 donors had all given proof of their fertility by having children during the last year, the only exceptions being 2 cases, in which the time was two years. Seven had more than one child and ten had pregnant wives. They had all been healthy during the last year, and had no high fever during this time. Their ages can be studied in the graph of Fig. 3, and the intervals in days between the ejaculations giving the samples and those preceding are given in Fig. 4. The sample was obtained either by manipulation or *coitus interruptus*, the former method being used in 15 cases and the other in the remaining 12. The time between the ejaculations and the examinations were noted and can be studied in Fig. 5.

It is probable that, even from this group of donors, pathological sperm samples were sometimes obtained, and this must be remembered when the properties of the samples are discussed.

The second group from 72 more or less pathological donors consists of 77 samples. The ages of the donors are given in Fig. 3, the intervals in days since the last ejaculations in Fig. 4, and the times in hours between the ejaculations and the examination of the samples

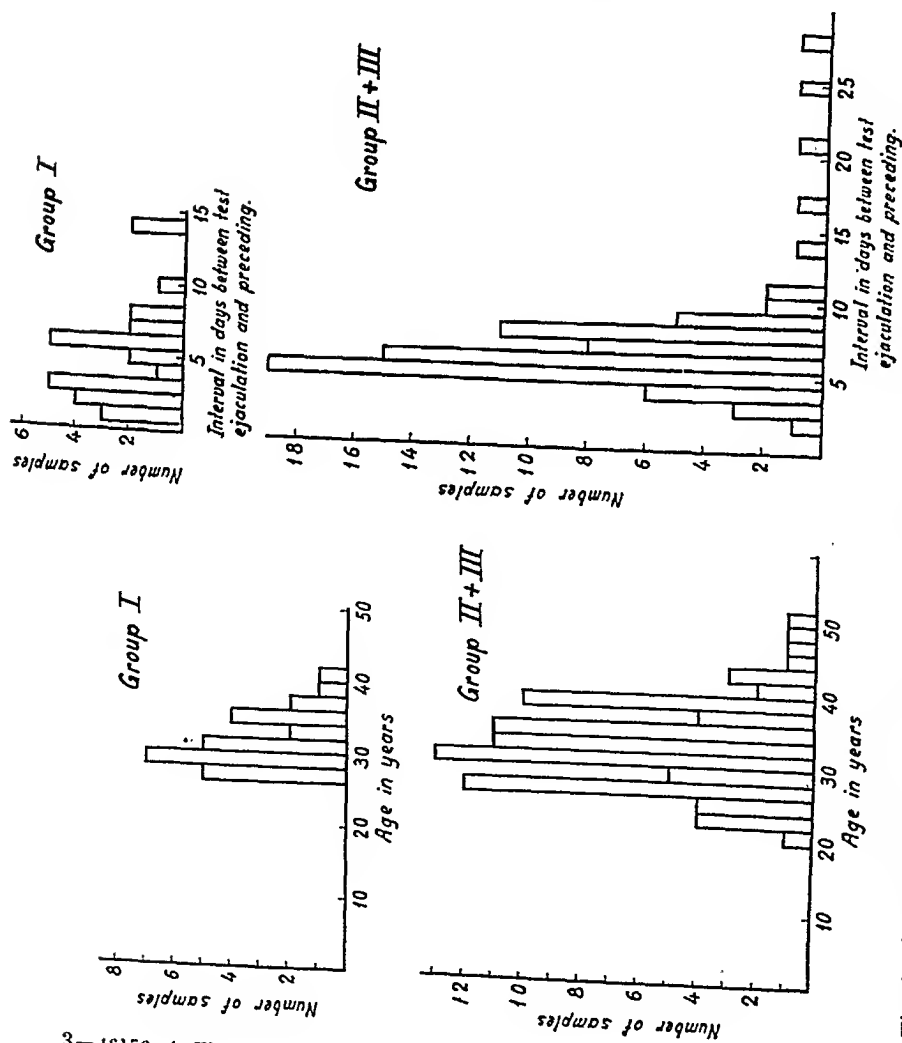


Fig. 3. Age of the donors of the samples.

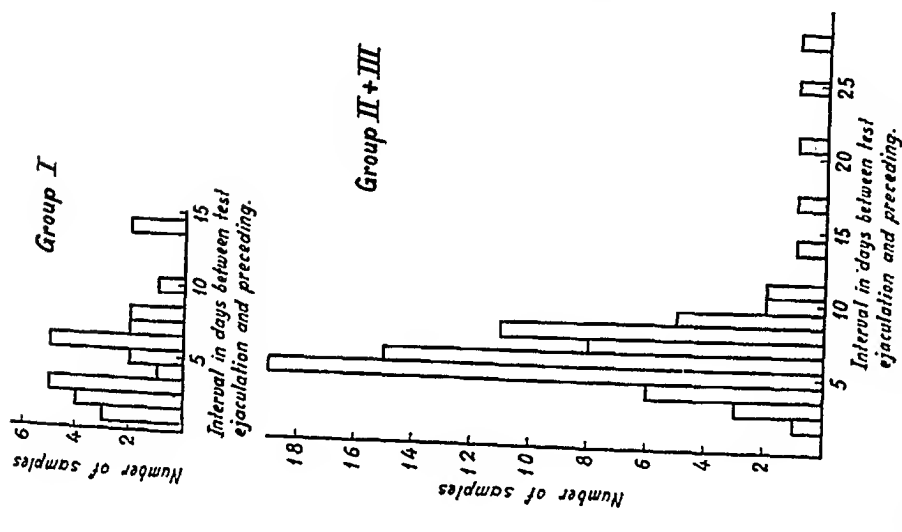


Fig. 4. Interval in days between testejaculation and preceding.

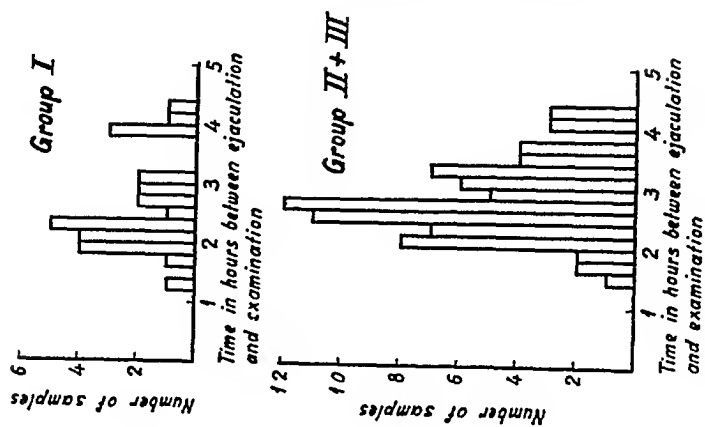


Fig. 5. Time between ejaculation and examination.

in Fig. 5. 16 samples were obtained by manipulation, 61 by *coitus interruptus*.

From the viewpoint of sterility the second group is very heterogeneous. As mentioned before, sterility depends on the breakdown of the physiological intercourse between two individuals, either of whom may be totally responsible for the failure. Almost all of these patients and their partners had been examined by the usual routine methods for the elucidation of sterility; in some cases pathological conditions had been found which gave ample cause for marital sterility. In cases where, for instance, radiograms had shown total obturation of both tubes in the female, and the male partner gave no signs of any pathological condition in his case-history or at the examination, it was considered less likely that the sperm samples from the male would be pathological.

In this way the group was divided into two sub-groups, one comprising 11 probably fertile patients, whose partners had pathological conditions causing sterility, and the other comprising the majority of cases, where it was impossible to draw conclusions from the clinical background of the samples.

The samples were placed in the former group when the men from whom they had been obtained were physically normal, their partners having one or more of the following pathological conditions: radiologically verified obturation or extensive adhesions of both tubes, chronic cervicitis, salpingo-oophoritis, or ovarian insufficiency as evidenced by microscopical examination of the uterine mucous membrane.

Unfortunately the examination of the sterile couples had been done by several different physicians and their methods had varied a great deal; excessive emphasis must therefore not be placed on the grouping. It is probable that many samples in the group obtained from men of uncertain clinical background were quite normal. Ross et al. (1941) in their investigation divided the donors into three groups: 1.) fertile, 2.) probably fertile, and 3.) probably sterile. Owing to the uncertainty in the clinical investigation in the present cases, the author has not been so definite in his opinions regarding the clinical characteristics of the groups. The samples were considered to be obtained from three categories of men:

- I. probably fertile (voluntary) donors,
- II. probably fertile patients with presumably sterile partners, and
- III. patients of uncertain clinical background.

## CHAPTER V.

### Investigations Concerning the Suspension of Spermatozoa.

#### The Concentration of Spermatozoa in the WARBURG Flasks.

The concentrations of spermatozoa in the WARBURG flasks were determined according to the method of CLARE HARVEY (see p. 23). The results of the determinations are reproduced in Fig. 6.

The concentration of spermatozoa in the flasks is conditioned by the original concentration of spermatozoa in the sample and the resuspension after the centrifugation. The values therefore have only a limited clinical interest but they have been cited so that they may be available for discussion in conjunction with the values obtained in the metabolic determinations.

Pathological conditions in the male genital organs often cause a reduction in the concentration of spermatozoa in the sample. When the various groups of samples are compared in Fig. 6, an obvious difference can be observed between groups II and III. None of the samples from group II has a concentration less than 0.50, expressed, as elsewhere in this work in units of  $10^8$  cells. In group III 65 per cent of the samples have this low concentration. Group I has intermediate figures. As stated above, the concentration of the cells in the WARBURG flasks is not solely dependent on the concentration of the original sample, but it is probable that this difference between the groups results chiefly from the original concentration in the sample.

#### The Determinations of the Percentage of Motile Spermatozoa.

The percentage of motile spermatozoa was determined according to the method of CLARE HARVEY (see p. 23). The results of the determinations are recorded in Fig. 7.

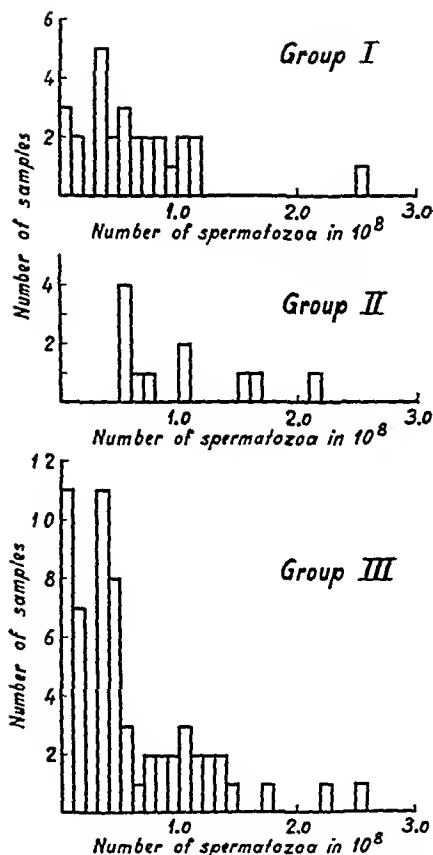


Fig. 6. Number of spermatozoa in WARBURG flask.

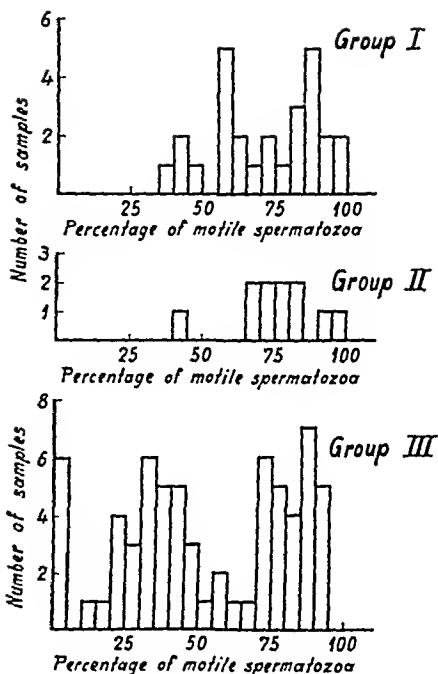


Fig. 7. Percentage of motile spermatozoa after the WARBURG determination.

In these diagrams a difference can be found between the groups. Groups I and II are similar with a high percentage of motile cells, no samples having a percentage lower than 35. The samples from group III are more evenly distributed over the values from 0 to 100 per cent and no less than 21 of the 66 samples of this group have below 35 per cent motile cells. The mean values for the percentages of motile spermatozoa in the groups are:

I.....	$71.7 \pm 3.4$	per cent
II.....	$78.4 \pm 4$	„ „
III.....	$52.8 \pm 4$	„ „

A statistically significant difference between the mean values of group III on the one hand and group I and II on the other can be demonstrated (see Table 5).

Table 5.

Comparison between the percentages of motile cells before and after the WARBURG determinations.

	Mean values of group			Differences between the mean values of the groups							
	I	II	III	I—III				II—III			
				$M_I - M_{II}$	$n$	$t$	$P$	$M_{II} - M_{III}$	$n$	$t$	$P$
Before	51.3	53.0	42	9.3	93	2.10	<0.05	11	77	1.76	<0.10
After	71.7	78.4	52.8	18.9	93	3.18	<0.01	25.6	77	2.92	<0.01

$M_I$ ,  $M_{II}$  and  $M_{III}$  are the mean values of the groups,  $n$  = the number of determinations made, and  $t$  and  $P$  have the usual significance, as employed in FISHER's work.

It is interesting that the heterogeneity of group III, already referred to in the discussion of page 34, is displayed here in the analysis of the motility of the spermatozoa from this group. It must be concluded from these determinations that the sperm samples with the highest figures for the percentage of motile cells, and thus resembling those from groups I and II, were obtained from the men in this group who had a higher fertility than the others. Conversely, we must suspect the donors of samples with low percentages of motile cells to be sterile.

Determinations of the motility of spermatozoa for clinical purposes have usually been made immediately after the delivery and liquefaction of the sperm sample at the laboratory, by the methods referred to on p. 18. The results of the determinations and their value for the appraisal of the sperm sample have been valued differently (see p. 12). Determinations of "longevity" (see p. 12) seem to have been more highly valued. Both these methods of studying the spermatozoon motility differ from that used in the present work.

In this investigation the determination of the percentage of motile cells was made after centrifugation and resuspension and after the WARBURG determination. The spermatozoa had been moving at body temperature, separated from their own plasma in a buffer solution of pH 7.3, for at least three hours before the examination was performed.

It has been demonstrated by MUSCHAT (1926) that the activity of the spermatozoa is highly influenced by the pH of the solution



in which they have been placed. It has also been shown, e. g. by KILLIAN (1933), that the pH of a sperm sample shows variations if it is unprotected from evaporation (see p. 11). Determinations of the

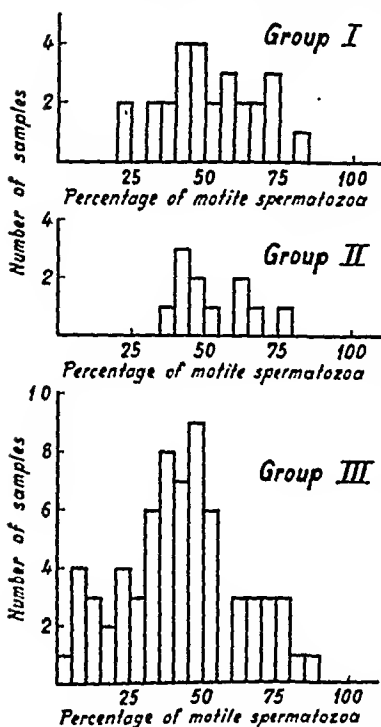


Fig. 8. Percentage of motile spermatozoa before the WARBURG determination.

pH in unprotected sperm samples have also given higher figures than in protected samples. It is therefore probable that determinations of the sperm-cell motility in samples of unphysiological pH, have given too low figures.

The writer has had the opportunity of studying the figures obtained in motility determinations when the samples were received at the laboratory of the Caroline Hospital and examined with the methods mentioned on page 18. Here the existence of differences between the motilities of the groups could already be suspected, as will be clear from Figs. 7 and 8, and in Table 5. It was impossible, however, to verify this statistically. In contrast with the results obtained after the WARBURG determinations, the figures for the different groups are too similar. Furthermore, when the motilities of the samples of group

III are studied, the heterogeneity of this group is not displayed.

It is not clear how the special circumstances of the present determinations have brought about this distinction between the clinical groups. Two causes are conceivable. Firstly, the method may have provided the spermatozoa with more physiological and identical surroundings than has hitherto been achieved. Secondly, the procedure may be considered as constituting a test for the "staying power" of the spermatozoa, viz. their longevity. It must be remembered that the cells had been stored for three hours at body temperature, which would have increased their metabolic rate appreciably above the low value at room temperature. The damaging effect of centrifugation and resuspension, as mentioned on p. 22, must also be considered.

It seems most likely that the qualitative difference between the clinical groups found in the determinations can best be explained by a combination of these different factors. Special emphasis should perhaps be laid on the fact that the spermatozoa had been placed in more identical and physiological surroundings than have previously been realized in similar determinations of spermatozoon motility for clinical purposes.

It has been stated, for example by HUFFMAN (1941), that there is a correlation between the amount of spermatozoa in a sample and their degree of motility, a high concentration usually being associated with good motility and vice versa. As these two properties of the suspensions had been measured, it was of interest to establish whether any support for this conception could be had. On a statistical analysis of the figures for the spermatozoa concentrations and motilities, no correlation was found in groups I and II, but in group III a statistically significant correlation could be calculated ( $r = 0.51$ ,  $P < 0.01$ ). The absence of any correlation between these two factors in groups I and II can probably be explained by the absence of any samples with low motility figures in these groups. On the other hand, the correlation in group III supports the view that the determination of both factors is of value for the evaluation of the properties of a sample. The existence of such a correlation could also be suspected, the two factors having been found to display typical variations in samples from men of different fertilities.

### The Formation of Lactic Acid.

The formation of lactic acid was determined according to the method of BARKER and SUMMERSON (1941, see p. 27). The result can be studied in Figs. 9 and 10, where the formation of lactic acid in micromoles per hour is plotted as a function of the number of spermatozoa present in the suspension.

It has been assumed by ROSS, MILLER, and KURZROCK (1941) that the formation of lactic acid in a suspension of spermatozoa is proportional to their number. An attempt has been made to prove this assumption statistically on the basis of the data given in Figs. 9 and 10. A significant coefficient of correlation was found in groups I and II:  $r = 0.85$ ,  $P < 0.01$ , and in group III:  $r = 0.58$ ,  $P < 0.01$ . In these calculations the values for concentrations of spermatozoa above 1.1 have been omitted, since these were comparatively few

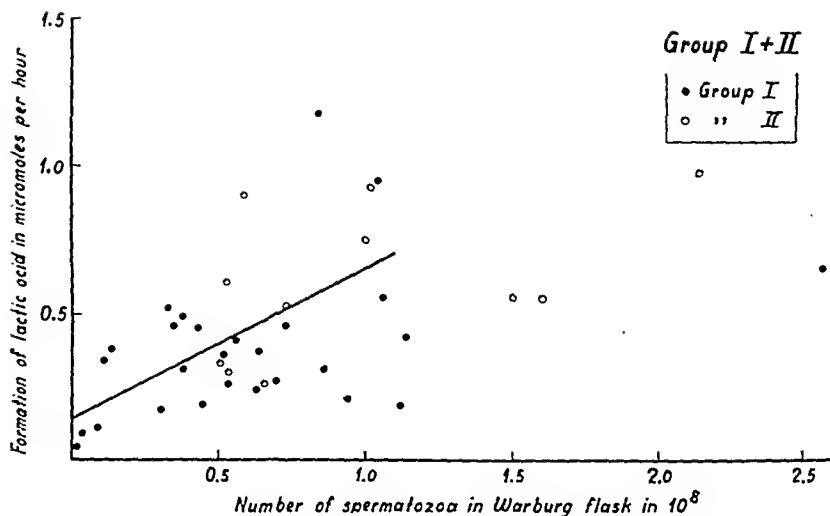


Fig. 9. Formation of lactic acid, Group I and II.

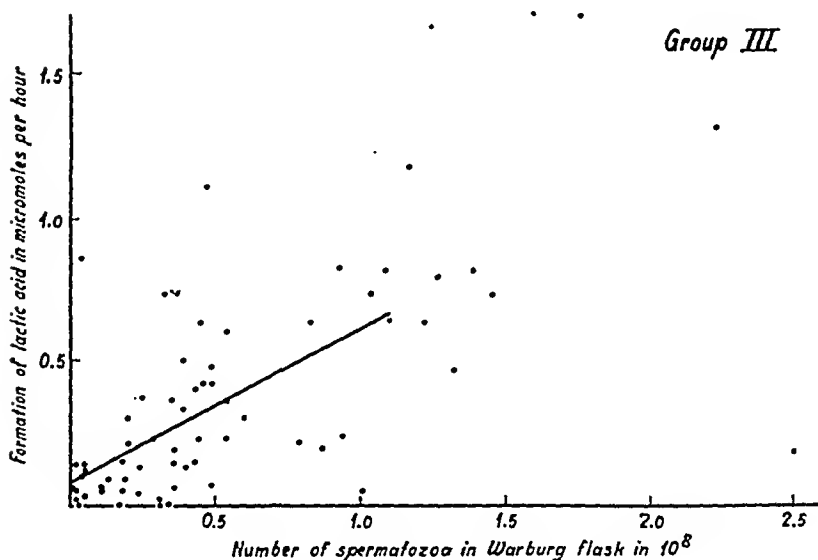


Fig. 10. Formation of lactic acid, Group III.

and therefore statistically more unreliable. The regression lines have been indicated in the figures:

$$\text{group I and II: } y = 0.52x + 0.14$$

$$\text{III : } y = 0.53x + 0.08,$$

where  $x$  = the concentration of sperm cells in  $10^8$ , and  
 $y$  = the formation of lactic acid in micromoles per hour.

During the determinations, the impression was obtained that the degree of motility was correlated with the formation of lactic acid by the cells. This correlation has also been assumed by Ross et al. (1941). Samples of high motility seemed to have a higher metabolic rate than those with a low percentage of motile cells. An opportunity now presented itself of proving this assumption. If it were correct, it would imply that the samples with relatively high figures for their formation of lactic acid would have correspondingly higher percentages of motile spermatozoa and vice versa. The mean value for the percentage of motile cells was therefore calculated for the group of samples above the regression line mentioned above (see Figs. 9 and 10) and compared with the corresponding value for the group below the line. The first group had formed relatively more and the second group relatively less lactic acid than the average. No correlation could, however, be found between the motility and the formation of lactic acid in groups I and II, although in group III a statistically verified difference could be established. Here the mean value for the percentage of motile cells above the line of regression was 59.1 and below the line 38.9 ( $t = 2.89$ ,  $P < 0.01$ ). The lack of correlation in groups I and II was probably due to the fact that the motility in these groups displayed so little variation.

In view of the correlation of the motility of the spermatozoa with their lactic acid formation a comparison was made of the concentration of motile cells with the amount of lactic acid formed by the suspension. The graphs thus acquired are reproduced in Figs. 11 and 12.

The correlation coefficients for the values in these graphs are 0.45 for groups I and II and 0.65 for group III. Both are statistically significant. The regression lines have been indicated in the graphs:

$$\begin{array}{ll} \text{group I and II: } y = 0.68x + 0.21, \text{ and} \\ \text{III} & : y = 0.62x + 0.13, \end{array}$$

where  $x$  and  $y$  have the same meanings as on p. 40.

Attention was then paid to the possible differences between the rates of formation of lactic acid by samples for the various clinical groups. The values for the samples in the graphs were now studied with the purpose of finding any difference between the groups (Figs. 9, 10, 11, and 12). It is evident from both these pairs of graphs that the regression lines from groups I and II indicate a relatively

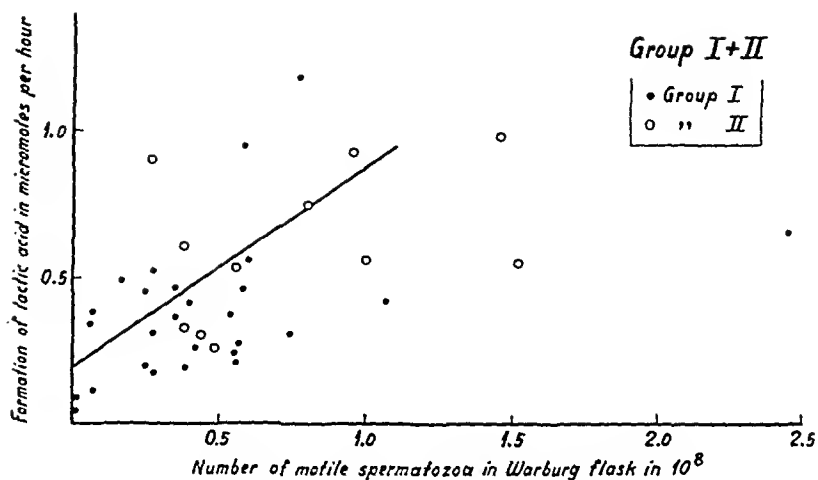


Fig. 11. Formation of lactic acid in comparison with the number of motile spermatozoa, Group I and II.

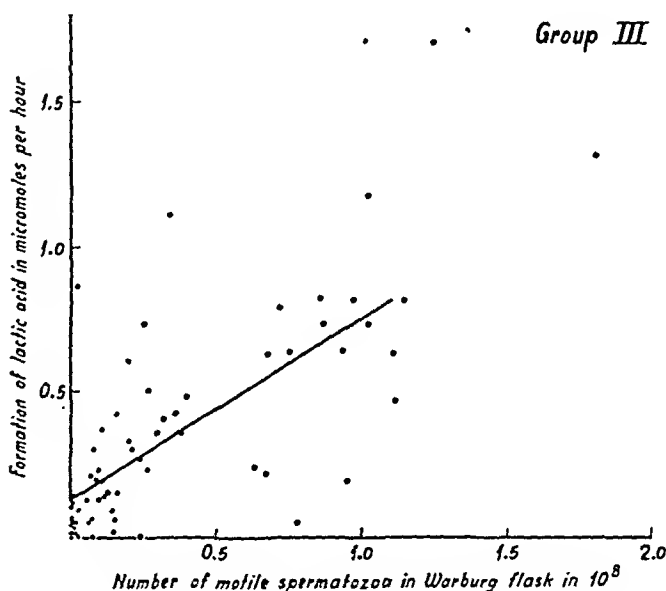


Fig. 12. Formation of lactic acid in comparison with the number of motile spermatozoa, Group III.

more active formation of lactic acid. When attempts have been made to establish the difference statistically, however, this has been found impossible because of the great variation and small number of the values in each group. In Fig. 13 the mean values have been calculated for the formation of lactic acid inside relatively broad ranges

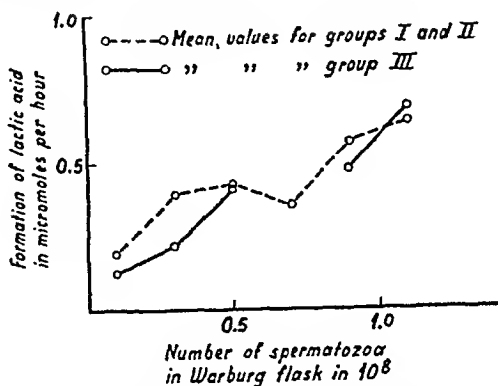


Fig. 13. Comparison between formations of lactic acid in different clinical groups.

of concentration in order to include as many samples as possible. Even this method has been unsuccessful in establishing any certain difference between the groups.

As mentioned on pp. 41 and 36, it has already been demonstrated that samples with a high motility usually have a relatively active formation of lactic acid and it has also been established that groups I and II have a higher figure for the mean percentage of motile cells. It was therefore probable that samples from groups I and II would have a more active formation of lactic acid than those from group III.

Such a tendency has been demonstrated, but the reason for the failure to establish a significant difference between the groups is not clear. It is probable that the methodical error is relatively greater in the more intricate determinations of the lactic acid formation than in the determinations of the density and motility of the suspensions of spermatozoa. It is possible that this accounts for the lack of success in confirming statistically the difference in the rates of lactic acid formation by samples from men of different grades of fertility.

### The Formation of Acid Metabolites.

The formation of acid metabolites was measured according to the method of WARBURG (see p. 25) and the resulting data are reproduced in Figs. 14 and 15.

The arrangement of the values in the figures resembles that in Figs. 9 and 10 where the formation of lactic acid was recorded. If the correlation is determined between the concentration of spermato-

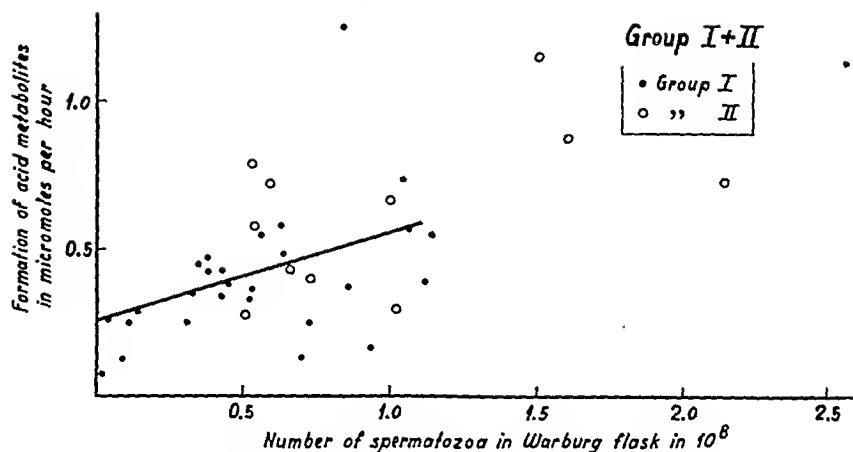


Fig. 14. Formation of acid metabolites, Group I and II.

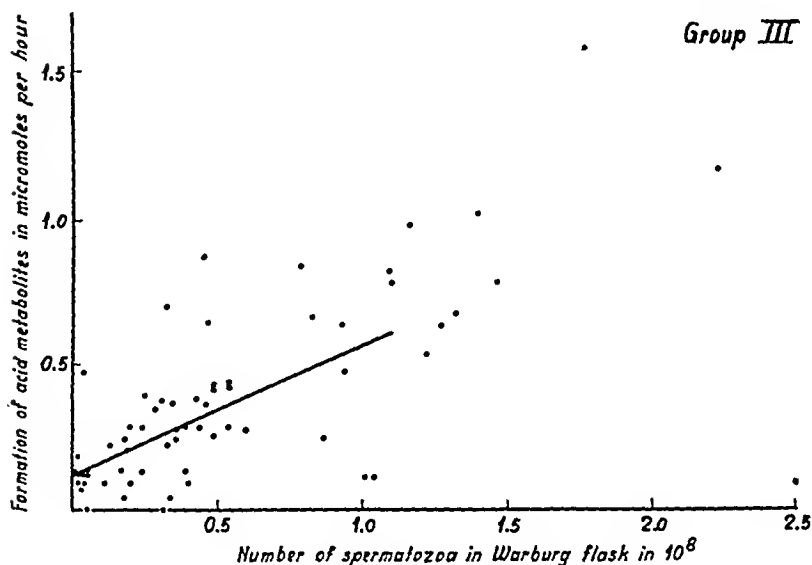


Fig. 15. Formation of acid metabolites, Group III.

zoa and the formation of acid metabolites the following values are obtained:

groups I and II:  $r = 0.39$ ,  $P < 0.05$  and  $y = 0.30x + 0.26$ ,  
 group III :  $r = 0.59$ ,  $P < 0.01$  and  $y = 0.44x + 0.12$ ,

where  $x$  and  $y$  have the same meaning as on p. 40. It will be seen from these figures that only a probable correlation exists between the density of the suspensions of spermatozoa and their formation

of acid metabolites in groups I and II, while the correlation is significant in group III.

It has been impossible here, in contrast with the case in the previous chapter, to find a significant correlation between the percentage of motile cells and the rate of formation of the metabolites. For the values of group III, however, such a correlation is probable. The calculation has been performed in the same manner as on p. 41 (the mean value for the percentage of motility above the regression line was 58.8, below 41.5,  $P < 0.05$ ).

A comparison of the mean values for the rate of formation of acid metabolites between the clinical groups shows that no certain difference can be established. The values inside each concentration range are so dispersed that no significant differences can be found.

The values for the quotient between acid metabolites and lactic acid formed by the spermatozoa in groups I and II and group III are given in Fig. 16. It is evident that

they show a wide variation, no significant difference appearing in this respect between the groups. The mean value for all quotients is  $1.36 \pm 0.17$ , which makes it evident that more acid metabolites than lactic acid are usually formed by the suspensions. The error in the mean to be expected from a combination of the corresponding errors in the two methods is only 0.04, viz. less than 25 per cent of the value actually found.

The variation between the values obtained with the two methods

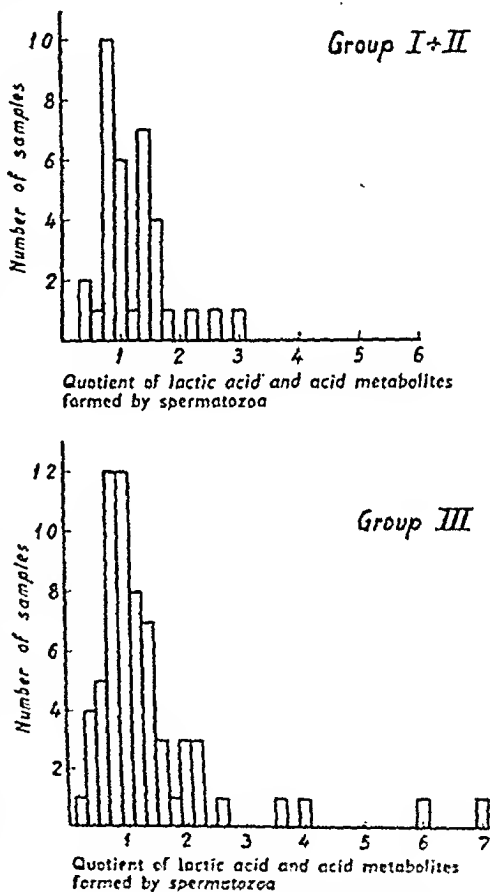


Fig. 16. Quotient of lactic acid and acid metabolites formed by spermatozoa.



has been so great that the WARBURG method must be considered unsuitable for this special purpose. It must be remembered that this method only measures the formation of acid metabolites, in contrast with that of BARKER and SUMMERSON which measures specifically the formation of lactic acid.

### The Consumption of Reducing Substance.

The consumption of reducing substance by the spermatozoa was measured according to the method of HAGEDORN-JENSEN (see p. 29). The resulting values can be studied in Figs. 17 and 18.

In these graphs it is not fully evident that the consumption of reducing substance (glucose) by the suspension of spermatozoa is proportional to the number of cells present. When the diagrams are treated statistically, the following values were obtained:

for groups I and II:  $r = 0.23$ ,  $P < 0.10$ ,

group III :  $r = 0.44$ ,  $P < 0.01$ ,  $y = 0.26x + 0.05$ ,

where  $x$  and  $y$  have meanings as on pp. 40 and 41. These figures show a probable lack of correlation in groups I and II, while in group III the correlation is significant.

In many samples, especially those with low sperm-cell concentrations, there was more reducing substance after the WARBURG

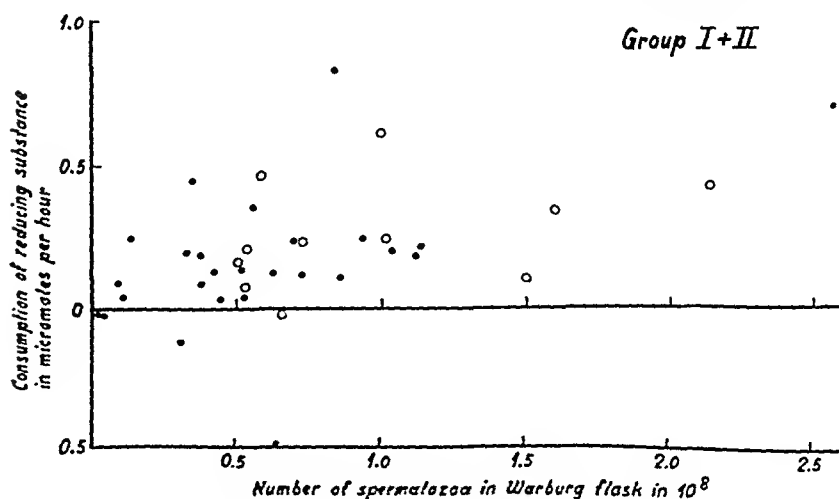


Fig. 17. Consumption of reducing substance, Group I and II.

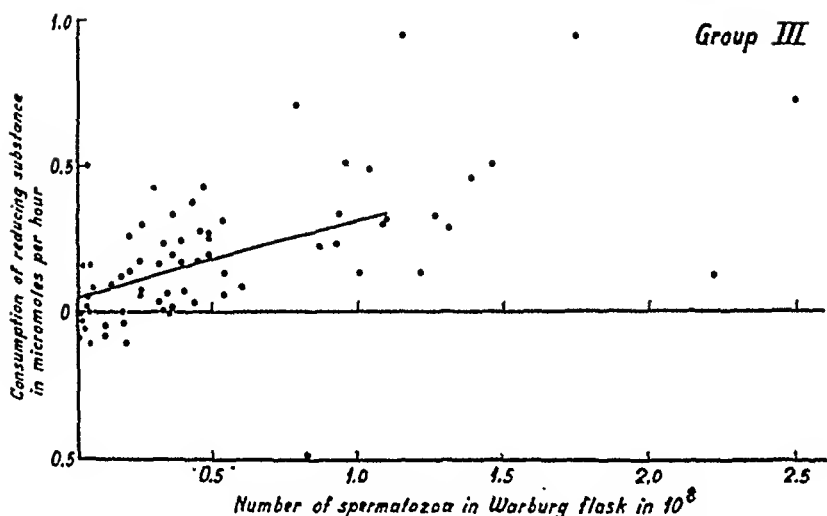


Fig. 18. Consumption of reducing substance, Group III.

determinations than before. This seems to indicate that reducing substance is formed during the determination and that this process is only partially counterbalanced by the consumption of reducing substance by the sperm-cells.

It has not been possible to find a higher consumption of reducing substance in the samples with high percentages of motility, which constitutes evidence against the statement by BERNSTEIN and SLOVOHOROV (1933) that the activity of the spermatozoa is highest when the curve of glucose-splitting is steepest. The present result is, however, the most likely in the circumstances, when the figures for the effect of repeated centrifugations are taken into account (see p. 22). In these experiments the consumption of reducing substance by the spermatozoa proved to be independent of the degree of motility.

The mean values for the samples of the clinical groups have been calculated in the same way as for lactic acid and acid metabolites. No significant differences could be found between the groups.

## CHAPTER VI.

### The Metabolism of the Plasma Samples.

The metabolism of the sperm plasma was studied in conjunction with that of the spermatozoa, as it proved practically impossible to separate the spermatozoa from the plasma without damaging the former in the process. A small amount of plasma, estimated to be about 0.2 ml or less, was therefore allowed to accompany the spermatozoa after the centrifugation and decantation and to mix with the new suspension fluid. It was impossible to determine whether this plasma had a metabolism which would materially interfere with the determinations on the spermatozoa. It was therefore decided to make determinations of the plasma metabolism by the same methods as were used for the spermatozoa.

#### Formation of Lactic Acid by Sperm Plasma.

The formation of lactic acid by the sperm plasma proved, with a few exceptions, so small that the interference with the values for the sperm-cell metabolism must have been of little significance. The mean formation of lactic acid per ml plasma and hour was the same for groups I and II and group III, viz.  $0.08 \pm 0.03$  with a range of variation from  $-0.20$  to  $+0.40$  micromoles lactic acid. In about half of the cases the amount of lactic acid increased during the period of examination; in about a third it decreased and the remaining cases showed no change in the concentration. No difference could be found in the amounts consumed or produced by the samples from the various clinical groups. When this fact was established the determinations were abandoned.

#### Formation of Acid Metabolites by Sperm Plasma.

The formation of acid metabolites was somewhat greater than that of lactic acid, but here also the possible disturbance in the values

Table 6.

Retention of CO<sub>2</sub> in plasma samples.

Clinical group	Mean value for the formation of acid metabolites per ml and hour in micromoles	Mean value for the formation of lactic acid per ml and hour in micromoles	Percentage of samples in this clinical group with retention of CO <sub>2</sub>
I + II .....	$0.23 \pm 0.02$	$0.08 \pm 0.04$	45
III .....	$0.25 \pm 0.01$	$0.08 \pm 0.02$	36

for the sperm metabolism was unimportant. The mean values for the formation of acid metabolites per ml plasma and hour was for groups I and II:  $0.23 \pm 0.02$ , and for group III:  $0.25 \pm 0.01$ . When the formation of acid metabolites was compared with that of lactic acid from the same sample, a slight difference was observed between the clinical groups. Groups I and II had relatively more samples showing a "retention of acid" by having lower figures for the acid metabolites than for the lactic acid. The results are shown in Table 6.

For a more detailed study of the difference between the formations of acid metabolites by the plasma and the spermatozoa, another fact must be mentioned. The formation of acid proceeded at a constant rate throughout the determination on the spermatozoa, but the WARBURG values for the plasma often showed a decrease towards the end of a run. The difference will be seen on a study of Fig. 19.

This change in the metabolic rate of the plasma seems to indicate that a metabolic process of a nature different from that in the spermatozoa proceeds in the plasma. The course of the metabolic rate does not resemble that of micro-organisms in the plasma. If bacteria

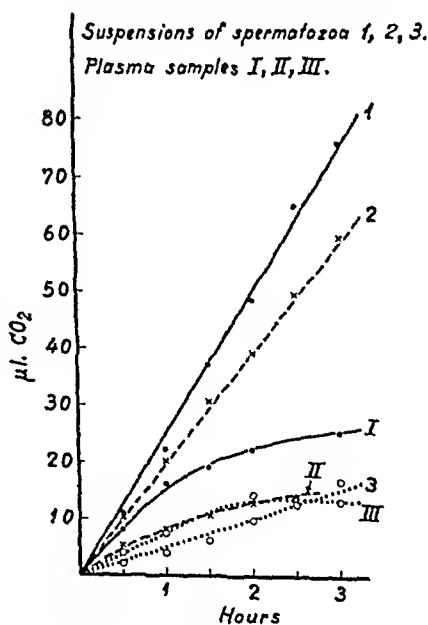


Fig. 19. Rates of formation of acid metabolites by spermatozoa and plasma.

had been metabolically active, a logarithmically increasing formation of acid would have occurred (see Fig. 2).

### Changes in the Concentration of Reducing Substance in the Plasma.

The consumption or formation of reducing substance in the plasma during the determinations varied without any apparent rule. The majority of the samples, 75 per cent of groups I and II and 64 per cent of group III, showed more reducing substance after the determination than before. No clinical differentiation has been found in the metabolic properties of the samples, as shown by the following figures. The mean changes in the concentration of reducing substance in the plasma samples were for groups I and II:  $+0.13 \pm 0.03$  micromoles per ml and hour and for group III:  $+0.12 \pm 0.03$ .

At the time of these determinations the author was not aware of the work by GOLDBLATT (1935) which had established the presence of diastase in human semen. This increase in the concentration of reducing substance in the plasma could therefore not be attributed to the action of any known enzyme in this fluid except hyaluronidase. It was suspected that hyaluronidase was responsible, since one of the methods for measuring hyaluronidase activity involves the determination of the increase in the reducing substance in the substrate. Hyaluronidase has been detected in sperm plasma, e. g. by CHAIN and DUTHIE (1940). The presence of a diastatic enzyme in the plasma was to be suspected from the fact that these are rather common in the fluids of the body. In an attempt to prove the activity of such an enzyme during the determinations, the following experiments were undertaken.

As a standard of the production of reducing substance a part was employed of a sample that had been treated in exactly the same way as the plasma samples in the series of clinical determinations. The concentrations of reducing substance before and after the three-hour WARBURG run were estimated as usual and the difference between the two was used as the standard. It was compared with the formation of reducing substance in samples to which had been added substrate in the form of hyaluronic acid, starch or glycogen. The concentration of these substrates was kept at 0.3 per cent in the solution in the WARBURG flask. The resulting values are given in Table 7.

Table 7.  
Enzymic activity in plasma.

Number of sample	Plasma volume in ml	Reducing substance in micromoles formed in			
		standard	hyaluronic acid	starch	glycogen
896	0.7	0.67	1.88	> 10	1.11
901	0.5	0.17	0.55	1.11	0.27
906	0.33	0.05	2.00	1.90	1.83
909	0.19	0.94	1.30	16.2	2.15
915	0.5	0.28	0.45	9.6	3.9
925	0.2	0.50	1.08	1.02	1.39
934	0.2	1.22	1.56	2.20	2.32

The presence of hyaluronidase and diastase was proved in six cases out of seven. The starch-splitting enzyme was also active in the splitting of the glycogen. It was interesting that the single case showing no hyaluronidase was also without diastase. The pH of the solution in which these reactions had taken place was 7.3, a value permitting full activity for diastatic enzymes. The starch-splitting enzyme of GOLDBLATT (see p. 10) had been active at 6.6 and 5.8 but not at 8.0. Curiously enough its activity was not studied at the normal pH of human sperm samples.

When these enzymes had been detected, it was of interest to find if their substrates also occurred in the plasma samples. The presence of hyaluronic acid was difficult to prove without special apparatus for this purpose and it was therefore decided not to study this question. Six samples were, however, analyzed for glycogen. The amount of glycogen was determined according to the method of SJÖGREN et al. (1938) and it was found in all six samples in concentrations ranging from 1 to 4 mg per ml plasma; at least 5.6 micromoles glucose per ml could have been formed from these amounts.

A possible explanation of the phenomenon had thus been found. As far as the author is aware, the presence of glycogen in human semen has not previously been demonstrated. The problem of measuring the glucose metabolism of the sperm sample becomes even more intricate when the possible presence of glycogen and diastase must be recognized. The importance of glycogen as a possible source of energy for the spermatozoa has been stressed by KARASSIK (1927).

## CHAPTER VII.

### The Concentration of Reducing Substance in the Original Sperm Sample.

The concentration of reducing substance in the sperm sample on delivery was determined simultaneously with the other determinations, since it was possible that this value might have some influence

on the metabolic properties of the sample. The resulting data are given in Fig. 20.

It is evident that all groups have very similar values. The range of variation is somewhat wider for group III, but this can be explained by the greater number of samples in this group. The mean value for groups I and II is  $390 \pm 15$  mg per cent and for group III  $415 \pm 62$  mg per cent. It is obvious that no significant difference exists between the values for the clinical groups. The figures are higher than those reported by HUGGINS et al. (1942) and GOLDBLATT (1935), 295 and 250 mg per cent respectively, but agree well with those obtained by ROSS et al. (1941),  $399 \pm 97$  mg per cent for samples from fertile and probably fertile

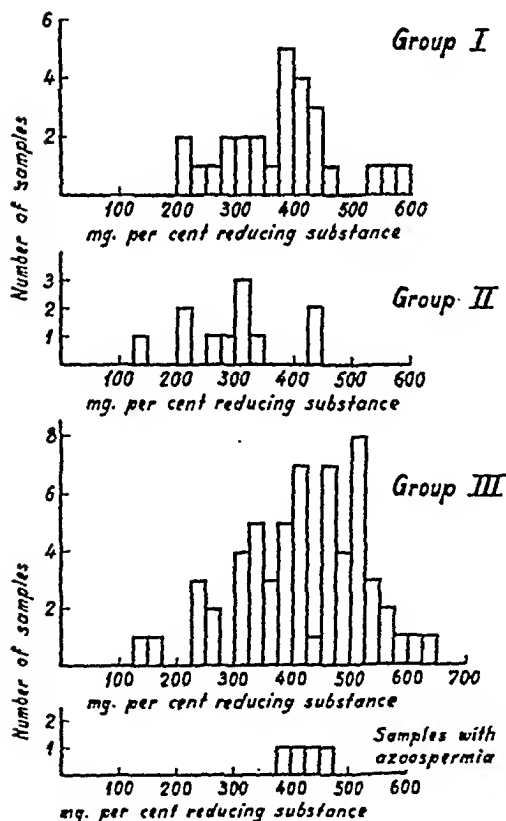


Fig. 20. Concentration of reducing substance in sperm sample.

men. The last-named authors found a higher figure (difference non-significant) for the samples from probably sterile men ( $508 \pm 159$  mg per cent) but no corroboration has been provided for this finding by the present material.

Similar values have been obtained from four samples showing azoospermia (absence of spermatozoa). This conflicts with the statement by MACLEOD and HOTCHKISS (1942) that samples with azoospermia have higher figures than the average sample.

Attempts have proved unsuccessful to find correlation between the metabolism of the sperm plasma and the concentration of reducing substance in the sperm.



## CHAPTER VIII.

### The Concentration of Lactic Acid in the Original Sperm Sample.

For the determination of lactic acid formation in a plasma sample, the concentration of this acid in the WARBURG flask was estimated before the experiment. From this value, which represented the amount of lactic acid in the aliquot of plasma placed in the flask, the concentration in the original sperm sample could be calculated, for the lactic acid must have been evenly distributed and unaffected by the centrifugation. The figures obtained are given in Fig. 21.

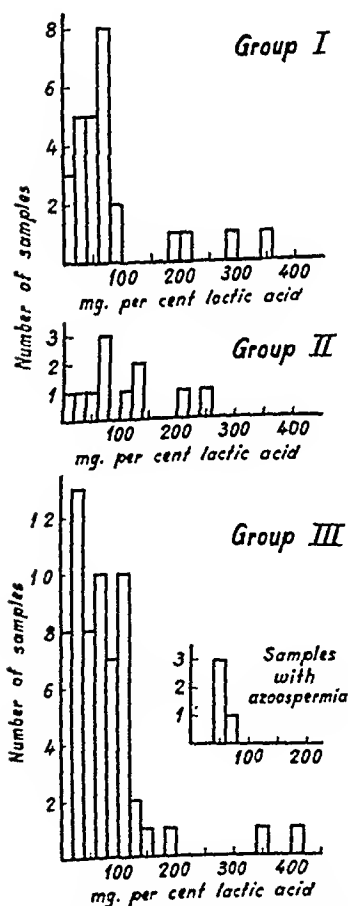


Fig. 21. Concentration of lactic acid in sperm sample.

Four cases of azoospermia were also examined and their lactic acid concentrations are of special interest. About 50 mg per cent lactic acid seems to be the concentration occurring in the sperm sample if no spermatozoa are present. The frequency curves in the diagrams are very asymmetrical, so only their median values have been calculated. These are for groups I and II: 67 mg per cent and for group III: 64 mg per cent. Thus no difference could be established between the clinical groups.

The values obtained show a wide range of variation and agree rather well with those reported by HUGGINS et al. (1942) and BERNSTEIN and SLOVOKOTOV (1933), 95 and 40—50 mg per cent respectively.

The relatively low values for the lactic acid concentrations in the four cases of azoospermia agree with the similar findings by MACLEOD and HOTCHKISS (1942).

An attempt was made to find signs of a correlation between the lactic acid concentration in the sperm sample and the concentration of spermatozoa, both motile and total. In contrast to the result of BERNSTEIN and SLOVOHOTOV (1933), however, no such correlation could be shown. Neither is there any correlation to be found with the formation of lactic acid and consumption of reducing substance by the sperm plasma, nor with the concentration of reducing substance in the sperm sample.

## CHAPTER IX.

### Influence of the Plasma Metabolism on that of the Spermatozoa.

Since small amounts of plasma, as mentioned on p. 21, were permitted to accompany the spermatozoa after the incomplete separation in the centrifugation and resuspension, it was of interest to investigate the possibility of correlations between the metabolic processes of the plasma and of the spermatozoa. If such a correlation were found, it would probably imply that the plasma metabolism disturbed the values for the sperm-cell metabolism. A statistical treatment was therefore made of the values for the formations of lactic acid and acid metabolites and the consumption of reducing substance by the spermatozoa, together with the corresponding values for the plasma. Before these figures were compared, each was referred to  $1 \times 10^8$  cells or 1.0 ml plasma.

No correlation was found between the formations of lactic acid or acid metabolites. A correlation was, however, suspected in groups I and II for the changes in the concentration of reducing substance. This was found to be statistically probable and almost significant for the values of group III ( $r = -0.30$ ,  $P < 0.02$ ). This suggests that high figures for the formation of reducing substance in the plasma correspond to low values for the measured consumption of reducing substance by the spermatozoa of the same sample. The probable explanation of this would appear to be that the small amount of plasma accompanying the spermatozoa, with its relatively active formation of reducing substance, compensates the loss of reducing substance caused by the spermatozoa. The actual consumption of reducing substance by the cells is therefore partly concealed by the plasma. When the plasma also consumes reducing substance, i. e. has a negative value for the formation, the loss of reducing substance is correspondingly greater.

Table 8.

Interference between the metabolic processes of plasma and spermatozoa.

Number of sample	Changes in the concentration of reducing substance in the plasma in micromoles per hour and ml	Value obtained for the consumption of reducing substance by $10^8$ cells from the same sample
607.....	+ 0.87	0.03
Mean value for all samples of group III .....	+ 0.13	0.22
612.....	- 0.78	1.45

The figures in the appended Table 8 may be studied as examples of this interference between the metabolic processes of plasma and spermatozoa.

## CHAPTER X.

### The Quotient of the Lactic Acid Formed and Glucose Consumed by the Spermatozoa.

One object of the investigation has been to find the quotient of the amounts of lactic acid formed and glucose consumed by the spermatozoa, since conflicting opinions have been held in this matter, as has been discussed on p. 14.

Several factors have, however, combined against the success of this determination. The most important was the disturbing effect of the plasma metabolism on the measurements of the consumption of reducing substance by the spermatozoa, as mentioned in the preceding chapter. Another factor was the inaccuracy of the method used for measuring the concentration of glucose. In order to circumvent these difficulties as far as possible, samples have been chosen with the smallest figures for the turnover of reducing substance by the plasma. Of these have been taken only those which showed consumptions of at least 0.15 micromoles reducing substance. As limits for the plasma metabolism have been selected  $\leq + 0.05$  and  $\geq - 0.05$  micromoles reducing substance per hour and ml.

On this basis 9 samples have been selected and the quotients calculated between lactic acid formed and reducing substance consumed. The resulting figures show a wide range of variation between 1.00 and 3.70, the mean value being  $2.50 \pm 0.74$ .

If one molecule of glucose is split and totally converted to lactic acid, two molecules of the latter are formed. The quotient would therefore be 2 when the concentrations in micromoles of lactic acid formed and glucose consumed were compared.

Owing to the uncertainty of these determinations, the problem is still unsolved whether the sperm cells form a proportionate amount of lactic acid as claimed by KILLIAN (1933), but it must be acknowledged that the results allow the possibility of this.

When the values of the quotient were compared for the samples from the clinical groups, no significant differences were found.

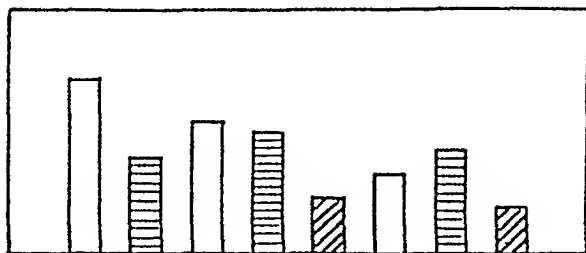


Table 9.

Correlation between age of donor and other factors and the properties of the sperm sample.

Number of sample	Interval between ejac. $\approx$ 10 days	Method of obtaining sample, x=manipul.	Age of donor $\approx$ 35 years	Time between ejac. and exam. $\approx$ 3 hours	Motile cells $> 40$ per cent	Conc. of reducing subst. $> 500$ mg per cent	Loss of reducing substance from sperm plasma	Concentration of lactic acid in sample $< 25$ mg per cent	$> 25$ per cent abnormal sperm cells (0=uninvestigated)
1	2	3	4	5	6	7	8	9	10
Group I									
I		x							0
II		x							0
III									0
598		x					x	x	x
IV		x							0
V				x					0
614		x					x		
629		x							x
636		x			x				
651									x
658		x	x						x
660	x		x	x			x		x
662			x	x					
677		x	x				x		
VI		x					x		0
VII	x	x	x	x					x
712							x		
713		x							
716	x			x					x
722				x					x
725		x					0		x
738									
757				x					x
773		x						x	x
776								x	
798		x							x
849							x		
Group II									
468			x						
487	x		x	x					
529			x	x			x		
558			x				0		
559	x								
609				x					

chief object in this experiment was to avoid the general poisoning of the animals which was produced in the experiments carried out by *Verzar* (subcutaneous injection of the drugs). This intention was realized by applying the poison directly in the intestine. In this way the intestine may be exposed to a relatively high concentration of the poison, and nevertheless a poor general condition of the animal is avoided.



□ *oleic acid.*      ▨ *controls.*

▤ *oleic acid + phlorrhizin.*

FIG. 8.

Radioactivity in phospholipids from rats intestine in which some of the loops were poisoned with phlorrhizin.

The experiment shown in figure 8 was carried out with the technique of a series of isolated loops of the intestine. The concentration of phlorrhizin applied was 0.02 molar (about 1 per cent) as used by *Carlsen* (1945) in work on amino acid and glucose absorption. In some of the isolated intestinal loops were injected an oleic acid emulsion, in others the same emulsion with the phlorrhizin added. The control loops contained pure sodium taurocholate solution.

On the narcotized rat, the loops were isolated and the solutions injected into them. It was assumed that after 15 minutes the absorption of the fatty acids would be running and that the poisoning would have developed in the loops containing phlorrhizin. At this point of time the  $^{32}\text{P}$  was injected intracardially, and 30 minutes later the animal was killed.

From the figure it is seen that the formation of phospholipids is of the same order of magnitude in the poisoned loops as in non poisoned, and in both of them considerably larger than in the controls. The formation of new phospholipids is, in this experiment, not prevented by phlorrhizin.



1	2	3	4	5	6	7	8	9	10
628	0				x				x
640		x	0	x	x	x			x
641						x			
648		x	x	x	x				x
649			x	x			0		
650			x						
664				x		x			
665									x
668			x			x	0		
669		x					x		x
670		x	x		x		x		x
684				x		0	x		x
696		x							x
700									x
701									x
703									
706			0	x	x	x	x		x
708								x	x
723			x						x
728							x		x
730		x			x		x		0
737		x	x			x		x	
749				x		x			
750		x				x		x	x
768	x		x			x	x	x	x
782		x	0			0	0	0	x
784		x			x		x	x	x
794									x

cept the last three, which had been obtained from a man recovering from an attack of orchitis caused by mumps and giving a complete azoospermia. His samples were examined when the spermatozoa began to reappear and they were interesting in that they all showed low values for the lactic acid concentration. With this exception the samples show variable figures; they have therefore been treated separately and not grouped in pairs. The donors all belonged to group III, men of uncertain fertility. For a comparison of the actual values obtained for samples provided at different times by the same donor, Table 10 should be consulted.

#### *Four Cases of Azoospermia.*

Four cases of sperm samples without spermatozoa, azoospermia, have been studied. As they are so few, little can be said of, and less concluded from, their metabolic properties. They have nevertheless been included in the material as they show an interesting sidelight of the sperm plasma metabolism (see pp. 52 and 54).

new phospholipids. Corresponding to this the specific activity is lower in the loops forming new phospholipids at the low rate.

From these experiments it will be seen that the rate of formation of phospholipids in the intestine is not depressed by phlorrhizin in a concentration which interferes heavily with glucose absorption.

#### Control experiments, absorption of glucose.

The main result of the experiments on absorption of oleic acid is that when the intestine absorbs oleic acid, phospholipids are formed at a considerably higher rate than in non absorbing intestine.

This increased rate of phospholipid formation may be due to a formation of phospholipids caused by the absorption of fatty acids, but another possibility cannot be excluded. When general absorption work is carried out, the metabolism of the cells increase and it might be suggested that this increase also interferes with the phospholipids. In other words, the turnover of phospholipids may follow an increase in the general metabolism of the intestine caused by absorption work.

If an increased activity gives increased phospholipid turnover, the absorption of glucose should give an effect similar to that of fatty acid absorption.

Experiments to investigate the effect of glucose absorption upon phospholipid formation, as compared with the effect of fatty acids, were carried out similar to the experiments described in the preceding chapter. In table 17 the results are given of an experiment in which solutions containing respectively oleic acid and glucose were injected into alternate loops of the small intestine.

The oleic acid emulsion was the same as that used in the experiments described above, 1 per cent oleic acid in 4 per cent Na-taurocholate solution. The glucose solution was a 0.15 molar (ca. 2.7 per cent) solution in 4 per cent Na-taurocholate (to make sure that no difference arises from a different bile acid content of the loops).

The figures in table 17 show that the total amount of phospholipids is the same in oleic acid absorbing and glucose absorbing loops. But the specific activity of the phospholipids is considerably higher in the loops absorbing oleic acid. The difference is about the same as that between oleic acid absorbing and non absorbing loops.

From this it will be seen that the phospholipid formation in glucose absorbing intestine is considerably lower than in fatty acid

## CHAPTER XII.

### Morphology of the Spermatozoa.

The data for the morphology of the spermatozoa have been obtained through Dr E. NORDLANDER, the head of the laboratory where the morphological properties of the spermatozoa were studied. For different reasons some samples were omitted from the analysis; of these one belonged to group III and six to group I. The figures have been given only for comparison with the metabolic data from the samples. No details are given concerning the method.

In accordance with the results of MACLEOD (1939), it was impossible to find any correlation between the metabolic properties of the samples and the morphology of the spermatozoa.

If the samples showing over 25 per cent of spermatozoa with abnormal forms of the head are counted in each clinical group, and these numbers are compared for all samples analyzed by this method, the figures in the accompanying Table 11 are found.

Table 11.  
Morphology of sperm cells.

Clinical group	Number of samples analyzed	Number of samples with over 25 per cent abnormal morphology	Percentage of samples in this group «clinically sterile»
I.....	21	12	57
II.....	11	1	9
III.....	66	37	56

According to the statements of MOENCH (1930) the donor of a sperm specimen is "clinically sterile" if more than 25 per cent of the spermatozoa show abnormally formed heads. Taking this as a criterion, group I is seen to include an astonishingly high number of "sterile" donors".

## CHAPTER XIII.

### Evaluation of the Methods Used for the Diagnosis of Male Sterility.

The object of this work has been to study the metabolism of spermatozoa and to find a suitable method for the differentiation of normal and pathological samples. The results of the various methods will not be recapitulated in detail, but a general survey may be useful to the reader.

Determinations of the degree of motility according to the method of CLARE HARVEY have shown decided differences in the properties of the clinical groups of samples. None of the samples from groups I and II had less than 35 per cent motile spermatozoa; in group III a third of the samples had these low figures. The differences between the mean percentages of motile cells in the groups were statistically significant. If the samples with less than 40 per cent motile cells were counted, group I showed one such sample in 27, group II none in 11, and group III 26 in 66.

The formation of lactic acid proceeded at similar rates in the different groups. Especially in the samples with low spermatozoon concentrations, however, a tendency could be established for the spermatozoa from group III to form smaller amounts of acid than those from the other groups. It must nevertheless be remembered that this was probably caused by the occurrence in this group, especially in the lower cell concentrations, of samples with low motility and hence a relatively weak formation of lactic acid.

The formation of acid metabolites as measured with the WARBURG method proceeded at similar rates in all three clinical groups and no clear difference could be established. This is consistent with the results of Ross et al. (1941) who, using a similar method, also found no difference in the rates of formation of acid metabolites by samples from donors of various degrees of fertility.

The consumption of reducing substance and the metabolism of the plasma were similar in all clinical groups. In contradiction to the authors mentioned above, but in accordance with the results of BERNSTEIN and SLOVOHOTOV (1933), no difference was found between the groups in the concentrations of reducing substance in the samples. The lactic acid concentrations were also practically the same.

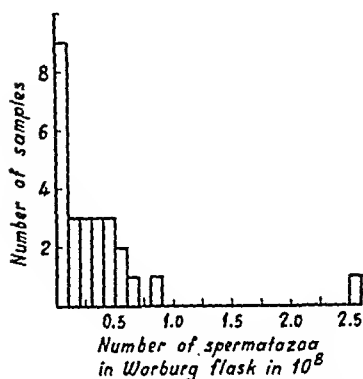


Fig. 22. Number of spermatozoa in specimens with low motility.

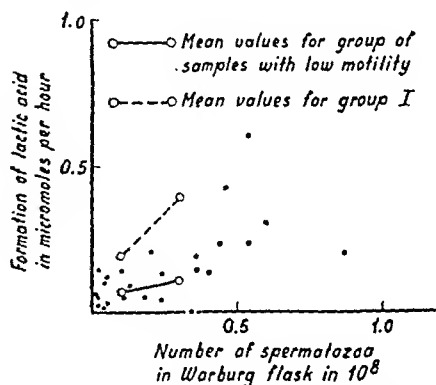


Fig. 23. Comparison between formations of lactic acid by groups of samples with different degrees of motility.

The values obtained by a morphological analysis of the spermatozoa were compared for the different groups and also with other metabolic properties of the samples. The results, however, were conflicting, and the method seemed uncertain, at least with respect to this material.

A study was made of the properties of the 26 samples from group III with less than 40 per cent of motile cells, which were therefore probably pathological and derived from sterile donors, in order to find what values suspectedly pathological samples gave with the methods employed in this investigation.

The concentration of the spermatozoa as compared with that in samples from fertile men is usually low, as can be seen in Fig. 22. The formation of lactic acid proceeds at a low rate when related to the total number of spermatozoa in the suspension. The figures for the formation of lactic acid and their mean values inside the concentration ranges concerned have been indicated for this group and for group I in Fig. 23.

The difference between the means is statistically significant (Conc. 0—0.2:  $t = 2.76$ ,  $P < 0.02$ ; conc. 0.2—0.4:  $t = 4.25$ ,  $P < 0.01$ ). As

mentioned before, this low rate of lactic acid formation can be explained by the low figures for motility in this group. No difference was found between the groups when formation of lactic acid was compared with the numbers of motile cells present.

No difference could be established between the data for this group and those from the other material when formation of acid metabolites, consumption of reducing substance, plasma metabolism, and

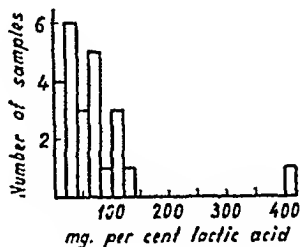


Fig. 24. Concentration of lactic acid in specimens with low motility.

the concentration of reducing substance in the sperm sample were compared. The lactic acid concentration in the sample was rather low, on the whole, with a median value of 53 mg per cent. There was, however, a wide range of variation, as can be seen from Fig. 24.

The morphological analysis showed 17 out of 24 samples examined (71 per cent) with more than 25 per cent of abnormally formed heads.

As a final impression from the results of these methods, it must be stated that the determination of the degree of motility, under the conditions employed here, has been the best method for differentiating the properties of the sperm samples. It has also given the results most consistent with the clinical grouping of the samples. 26 of the 66 samples in group III were indicated as probably pathological; determinations of the lactic acid formation by these samples confirmed that there was a statistically verified difference between the metabolic properties of these and the normal sperms.

## CHAPTER XIV.

### Summary.

The object of this investigation has been to find a biochemical method for establishing male sterility from samples of sperm.

Chapter I. After a brief review of the literature on the metabolism and motility of spermatozoa, the physico-chemical properties of human semen, and the few investigations in which the metabolic properties of the sperm sample have been used as criteria for the state of fertility of the donors of the samples, a description is given of the methods used in the present work.

Chapters II and III. The basic method used was the determination of the formation of acid metabolites by the spermatozoa according to WARBURG. The sample was centrifuged sharply in order to separate the spermatozoa from the plasma. They were resuspended in an isotonic buffer solution and placed in a WARBURG flask, the formation of acid metabolites being measured for three hours at body temperature. The method of CLARE HARVEY was used for the determination of the density of the suspension and the proportion of motile cells. These determinations were made after the sample had been investigated by the WARBURG method.

The changes in the lactic acid concentration were measured according to the method of BARKER and SUMMERSON. Conflicting opinions have been held as to whether the spermatozoa form a proportionate amount of lactic acid from the glucose that they consume. For the study of this question the consumption of reducing substance was measured (HAGEDORN—JENSEN). In order to estimate the metabolism of the sperm plasma and to detect any disturbing effect produced by the plasma metabolism on the measurement of the sperm metabolism, the metabolism of the sperm plasma was studied with the same methods as for the spermatozoa.

Chapter IV. The material to be investigated consisted of 104

samples. The donors of these samples were divided according to their degrees of fertility into three groups:

- I 27 samples from probably fertile men
- II 11 samples from probably fertile patients with presumably sterile partners, and
- III 66 samples from men of uncertain clinical background.

The samples from group I had been obtained from voluntary donors, the others from patients who had sought medical aid for marital sterility. The existence of pathological properties in the sperm samples would therefore be most likely in group III; groups I and II would probably be normal.

Chapter V. When the percentages of motile cells were determined, a difference could be observed between the clinical groups. Only one of the samples from groups I and II had less than 40 per cent motile cells; in group III more than a third of the samples had such low figures. The differences between the mean values for the percentage of motile cells in the groups were statistically significant. The results of determinations of the spermatozoon motility have been valued very differently for the diagnosis of male sterility and have often been considered to be of little use. The factors are discussed which have probably conditioned this judgment. The present results are compared with the motilities of the same samples before the WARBURG run. These figures are almost uniformly lower and show no significant difference between the clinical groups.

The lactic acid formation proceeded at a rate which was correlated to the number of spermatozoa in the suspension by a statistically significant coefficient of correlation. The degree of motility and the production of lactic acid were also correlated. When the mean values for the lactic acid formation were compared for the groups no significant difference could be found, but in more diluted suspensions a tendency could be established for the less fertile samples to form relatively less lactic acid than the others.

The formation of acid metabolites occurred at a rate similar to the lactic acid production, but like the consumption of reducing substance it was very variable and the above-mentioned correlations were impossible to find.

Chapter VI. The changes in the lactic acid concentration in the sperm plasma proved to be relatively insignificant. The formation



of acid metabolites was more active and the changes in the concentration of reducing substance in the plasma were also sometimes quite considerable. No clinical significance could be found in these properties. About two thirds of the samples showed an increase in the concentration of reducing substance after the determination. In an attempt to find a possible explanation of this fact, the author demonstrated the presence of an active diastase in six samples out of seven and that of glycogen in six samples out of six. The presence of hyaluronidase was shown in the samples with diastase.

Chapters VII and VIII. The concentrations of reducing substance and lactic acid were determined in the original sperm samples. No significant differences could be found between the clinical groups. The ranges of variation were wide.

Chapter IX. A statistically verified correlation was found between the changes of concentration of reducing substance in the plasma and the consumption of reducing substance by the spermatozoa in the same sample. This established that the metabolic activity of the plasma which accompanied the spermatozoa, must have disturbed the values obtained for the spermatozoon metabolism. No such correlations were found for the formation of lactic acid or acid metabolites.

Chapter X. In order to obtain as accurate values as possible for the quotient between lactic acid formed and glucose consumed by the spermatozoa, those samples were chosen which showed the smallest figures for the changes in the concentration of reducing substance in the plasma. The accuracy of the determinations of reducing substance consumed by the spermatozoa was increased by selecting those samples which showed the highest absolute figures for the consumption of reducing substance. The mean value of this quotient for nine samples was  $2.50 \pm 0.76$ , the amounts of glucose and lactic acid being expressed in micromole. This value is rather uncertain, but it renders probable an approximately proportionate formation of lactic acid in these circumstances.

Chapter XI. No correlation has been found between the properties of the sperm sample and the age of the donor, the method by which the sample was obtained, and the time elapsing between ejaculation and examination.

Chapter XII. No correlation has been found between the above-mentioned properties and the morphology of the spermatozoa.

## GENERAL DISCUSSION

The present investigation was carried out as an attempt to throw some light upon the chemical processes involved in fat absorption.

The most recent work on fat absorption (*Frazer*) shows that the absorption of fat may take place in the unsplit condition as minute droplets, but there is no doubt that the absorption of fat can also take place in the way that the fats are hydrolysed to fatty acids and glycerol and the components absorbed separately.

The fats are split by the lipolytic enzymes of the digestive juices. *Pflügers* theory was that the fatty acids then combine with the sodium bicarbonate of the pancreatic juice to form soaps. The fatty acids are in this way made water soluble and may be absorbed in this state. Later on it was stated that the reaction of the intestine was actually slightly acid, and *Pflügers* theory of soap formation was rejected. It was well known that soaps are split up when an acid is added, and it was taken for granted that no soap (or only infinitesimal quantities of soap) can exist at the pH of the intestine.

In the writers experiments it was shown that if an acid is added to a soap solution until the pH of the intestine is reached, there will still be a considerable fraction of the fatty acids present as soap. Therefore, the pH of the intestinal contents is not a hindrance to the absorption of fatty acids as soaps.

When fatty acids or soaps are introduced into the small intestine they are readily absorbed, esp. when bile acids are present. The bile acids have the property of keeping the fatty acids in aqueous solution. Therefore it has been supposed that the fatty acids are absorbed as a water soluble bile acid complex. This complex must be of considerable size because a number of bile acid molecules (3—8) is necessary for keeping one fatty acid molecule in

Number of sperm sample	Age of donor	Interval in days since last ejac.	Time in hours and minutes between ejac. and examination	Method of obtaining the sample (c = coitus interr., m = manipuli.)	Percentage of sperm cells with abnormal forms of head	Number of sperm cells in Warburg-flask in 10 <sup>5</sup>	Percentage of motile cells	Number of motile cells in Warburg-flask
1	2	3	4	5	6	7	8	9
GROUP I								
I	28	1	2 <sup>h</sup> 15'	m	—	0.38	44	0.17
II	33	3	2 <sup>h</sup> 10'	m	—	1.04	57	0.59
III	28	2	2 <sup>h</sup> 10'	c	—	0.14	49	0.07
598	28	3	2 <sup>h</sup> 30'	m	27	0.09	77	0.07
IV	30	1	1 <sup>h</sup> 20'	m	—	0.33	85	0.28
V	30	6	3 <sup>h</sup>	c	—	0.31	89	0.28
614	34	6	2 <sup>h</sup> 45'	m	6	0.35	99	0.35
629	28	3	2 <sup>h</sup>	m	64	0.04	40	0.01
636	29	2	2 <sup>h</sup> 15'	m	14	1.12	35	0.39
651	30	6	2 <sup>h</sup> 55'	c	33	0.45	55	0.25
658	37	4	1 <sup>h</sup> 50'	m	38	0.11	61	0.06
660	38	14	3 <sup>h</sup> 50'	c	31	0.94	60	0.56
662	41	8	3 <sup>h</sup> 50'	c	21	2.57	95	2.45
677	35	8	1 <sup>h</sup> 45'	m	15	1.14	94	1.07
VI	29	5	2 <sup>h</sup> 20'	m	—	0.73	80	0.58
VII	37	10	3 <sup>h</sup>	m	38	0.63	87	0.55
712	31	7	2 <sup>h</sup> 50'	c	11	0.53	80	0.42
713	34	1	2 <sup>h</sup> 20'	m	24	0.56	72	0.40
716	28	14	4 <sup>h</sup>	c	27	1.06	57	0.60
722	27	6	4 <sup>h</sup> 15'	c	50	0.86	86	0.74
725	27	3	1 <sup>h</sup> 30'	m	32	0.70	80	0.57
738	27	7	2 <sup>h</sup>	c	18	0.43	58	0.25
757	34	5	3 <sup>h</sup> 50'	c	32	0.52	68	0.35
773	33	2	1 <sup>h</sup> 50'	m	37	0.02	55	0.01
776	30	2	2 <sup>h</sup> 20'	c	19	0.64	85	0.54
798	26	3	2 <sup>h</sup> 40'	m	48	0.38	72	0.27
849	26	6	1 <sup>h</sup> 50'	c	18	0.84	92	0.77
GROUP II								
468	39	4	2 <sup>h</sup> 30'	c	18	0.54	81	0.44
487	39	21	3 <sup>h</sup> 30'	c	22	2.14	68	1.46
529	39	9	3 <sup>h</sup> 15'	c	14	1.00	80	0.80
558	38	8	2 <sup>h</sup> 35'	c	22	1.50	67	1.00

Number of sample	Lactic acid formed in micromol. per hour	Acid metabolites formed in micromol. per hour	Reducing substance consumed in micromol. per hour	Volume of plasma sample	Lactic acid formed by plasma in micromol. per hour	Acid metabolites formed by plasma in micromol. per hour	Reducing substance consumed in plasma in micromol. per hour	Concentration of reducing substance in sperm sample in mg per cent	Concentration of lactic acid in sperm sample in mg per cent
10	11	12	13	14	15	16	17	18	19
GROUP I									
I	0.49	0.47	0.19	1.00	+0.08	0.24	+0.05	414	55
II	0.95	0.74	0.21	1.00	+0.14	0.30	+0.59	459	66
III	0.38	0.20	0.25	1.00	+0.02	0.04	+0.17	308	72
598	0.11	0.12	0.09	0.33	-0.03	0.03	-0.26	500	9
IV	0.52	0.35	0.20	1.00	+0.25	0.07	+0.70	320	27
V	0.17	0.25	+0.12	1.00	+0.02	0.31	+	447	44
614	0.46	0.45	0.45	1.00	+0.13	0.22	minus	331	87
629	0.09	0.20	+0.03	0.40	-0.03	0.16	+	412	68
636	0.19	0.39	0.19	0.57	-0.02	0.06	+0.04	358	77
651	0.19	0.38	0.03	0.81	0	0.16	+0.30	595	196
658	0.34	0.25	0.04	0.80	+0.12	0.30	+0.27	396	56
660	0.21	0.16	0.25	0.40		0.06	-0.06	295	290
662	0.65	1.14	0.70	1.00		0.16	+0.34	385	340
677	0.42	0.55	0.22	1.00		0.24	-0.38	486	51
VI	0.46	0.25	0.12	0.33		0.04	-0.03	393	30
VII	0.24	0.58	0.13	0.67		0.18	+0.89	265	30
712	0.26	0.36	0.04	0.67		0.19	-0.05	430	200
713	0.41	0.55	0.36	0.62		0.15	+0.08	400	47
716	0.56	0.57	+0.00	1.00		0.09	+0.67	204	33
722	0.31	0.37	0.11	1.00		0.23	+0.53	385	88
725	0.27	0.13	0.24	1.00		0.35		567	79
738	0.45	0.34	0.13	1.00		0.22	+0.06	421	68
757	0.36	0.33	0.14	1.00		0	+0.04	348	61
773	0.04	0.07	+0.03	0.67		0.04	+0.11	232	6
776	0.37	0.48	+0.49	0.67		0.19	+0.24	292	5
798	0.31	0.42	0.09	1.00		0	+0.09	449	77
849	1.18	1.25	0.83	0.67		0.18	-0.16	204	30
GROUP II									
468	0.30	0.58	0.21	1.00	-0.30	0.03	+0.28	390	210
487	0.98	0.73	0.43	1.00	-0.13	0.47	+0.07	235	122
520	0.75	0.67	0.62	0.50	+0.17	0.19	-0.40	300	78
558	0.56	1.16	0.11	1.00	+0.18	0.28		534	79

1	2	3	4	5	6	7	8	9
559	31	14	3 <sup>b</sup> 25'	c	23	0.59	45	0.27
609	31	7	4 <sup>b</sup>	c	18	1.02	94	0.96
655	31	5	4 <sup>b</sup> 15'	c	27	0.51	75	0.38
678	23	5	2 <sup>b</sup> 40'	c	17	1.60	95	1.52
679	41	6	2 <sup>b</sup> 20'	c	23	0.53	72	0.38
729	32	6	2 <sup>b</sup> 45'	m	23	0.66	74	0.49
767	35	9	2 <sup>b</sup> 50'	c	19	0.73	77	0.56

## GROUP III

452	26	11	2 <sup>b</sup> 55'	c	42	0.40	24	0.10
473	33	6	3 <sup>b</sup> 45	c	53	0.39	51	0.20
474	25	5	2 <sup>b</sup> 45'	c	18	2.22	81	1.80
477	32	28	2 <sup>b</sup> 05'	c	17	2.50	38	0.95
478	28	3	2 <sup>b</sup>	c	34	0.79	86	0.68
481	28	8	3 <sup>b</sup> 15'	c	34	0.93	94	0.86
482	28	7	2 <sup>b</sup>	c	23	1.22	90	1.11
486	26	8	2 <sup>b</sup> 35'	c	54	0.94	68	0.64
492	32	6	3 <sup>b</sup> 15'	c	32	0.05	31	0.01
493	34	9	3 <sup>b</sup> 15'	c	35	0.04	41	0.02
536	31	6	3 <sup>b</sup> 35'	c	30	0.36	30	0.11
538	31	5	2 <sup>b</sup> 15'	m	12	1.27	57	0.72
539	43	6	2 <sup>b</sup> 40'	c	10	1.16	88	1.02
542	32	7	2 <sup>b</sup> 45'	c	20	0.33	77	0.25
546	34	5	1 <sup>b</sup> 40'	m	23	0.34	4	0
549	46	3	4 <sup>b</sup> 15'	c	18	0.49	73	0.36
554	32	5	2 <sup>b</sup>	c	30	0.46	35	0.16
561	26	6	2 <sup>b</sup> 55'	c	26	0.24	23	0.06
563	37	10	2 <sup>b</sup> 30'	c	21	1.46	71	1.03
564	31	10	2 <sup>b</sup> 40'	c	38	0.02	30	0.01
565	27	5	3 <sup>b</sup> 40'	c	24	0.20	40	0.08
569	38	11	2 <sup>b</sup> 45'	c	29	0.51	37	0.20
573	26	8	3 <sup>b</sup> 45'	c	45	0.20	32	0.07
576	37	5	2 <sup>b</sup> 15'	c	21	0.04	0	0
584	32	6	3 <sup>b</sup> 30'	c	13	0.13	21	0.03
585	33	4	2 <sup>b</sup> 20'	m	15	0.25	43	0.11
594	34	9	3 <sup>b</sup> 10'	c	40	0.95	25	0.01
596	39	3	3 <sup>b</sup> 20'	c	22	0.39	71	0.27
597	41	9	3 <sup>b</sup> 15'	c	18	0.24	0	0
599	35	4	2 <sup>b</sup> 35'	c	18	0.54	18	0.16
607	34	8	2 <sup>b</sup> 55'	c	35	0.36	35	0.12
612	36	7	3 <sup>b</sup> 45'	c	11	0.29	94	0.27
613	36	5	2 <sup>b</sup> 35'	c	18	0.43	75	0.32
615	27	4	3 <sup>b</sup>	c	13	0.49	48	0.24
616	39	5	4 <sup>b</sup>	c	49	0.18	87	0.16

10	11	12	13	14	15	16	17	18	19
559	0.90	0.72	0.47	0.67	+0.18	0.27	+0.02	428	79
609	0.93	0.30	0.25	0.67		0.22	+0.62	351	120
655	0.33	0.28	0.17	1.00		0.19	+0.11	419	76
678	0.55	0.88	0.35	0.50		0.11	+	530	255
679	0.61	0.79	0.08	1.00		0.27	+0.05	402	105
729	0.26	0.43	+0.02	0.67		0.19	+0.49	305	25
767	0.53	0.40	0.24	0.67		0.16	-0.20	417	0

## GROUP III

452	0.13	0.09	0.07	0.67	0	0	-0.15	306	400
473	0.33	0.13	0.24	1.00	-0.04	0.21	-0.08	404	340
474	1.31	1.17	0.12	1.00	-0.11	0.43	+1.30	423	111
477	0.19	0.09	0.71	1.00	+0.17	0.41	-0.13	479	94
478	0.22	0.84	0.70	1.00	-0.08	0.36	-0.28	480	94
481	0.82	0.63	0.23	1.00	-0.13	0.31	-0.08	404	45
482	0.63	0.53	0.13	1.00	+0.04	0.43	+0.94	423	119
486	0.24	0.47	0.33	1.00	-0.10	0.30	+	649	77
492	0.12	0.13	0.16	0.50	+0.05	0.11	-0.46		36
493	0.86	0.47	0.50	1.00	+0.03	0.14	-0.28		21
536	0.19	0.24	0.19	0.50	+0.04	0.03	-0.02	226	75
538	0.79	0.63	0.32	0.25	+0.03	0.12	+0.02	468	84
539	1.18	0.98	0.93	0.70	-0.02	0.19	+0.21	500	55
542	0.73	0.70	0.23	1.00	+0.08	0.41	+0.02	249	113
546	0	0.04	0.06	1.00	-0.08	0.31	+0.19	442	48
549	0.42	0.41	0.25	0.50	+0.06	0.17	-0.24	238	95
554	0.42	0.36	0.27	1.00	-0.03	0.27	+0.24	247	90
561	0.13	0.13	0.07	1.00	0	0.32	-0.35	494	58
563	0.73	0.78	0.50	1.00		0.27	+	320	100
564	0.14	0.09	0.16	1.00	-0.10	0.05		520	74
565	0.30	0.09	0.14	1.00	+0.22	0	+0.03	400	24
569	0.60	0.43	0.31	1.00	+0.11	0.43	-0.35	468	108
573	0.21	0.28	0.26	0.50	+0.12	0.21	+0.29		68
576	0.01	0.09	0.02	1.00	+0.24	0.36	+0.35	604	
584	0.09	0.22	0.09	0.50	0	0.07	+0.08	571	
585	0.37	0.39	0.30	0.67	0	0.15	+0.13	358	
594	0.03	0.12	0	0.67	-0.09	0.21	-0.21	410	23
596	0.50	0.28	0.17	0.67	-0.02	0	0	251	56
597	0.04	0.28	0.06	0.67	+0.17	0.13	-0.03	390	110
599	0.23	0.28	0.06	1.00	+0.23	0.12	+	517	67
607	0.14	0.27	0.01	0.44	+0.02	0.07	+0.38	251	50
612	0.23	0.34	0.42	0.33	+0.10	0.09	-0.26	156	66
613	0.40	0.47	0.37	1.00	+0.14	0.14		458	80
615	0.07	0.42	0.19	0.67		0.18	-0.26	380	102
616	0.15	0.24	0	0.33		0.07	-0.19	110	129

1	2	3	4	5	6	7	8	9
617	49	6	2 <sup>h</sup> 30'	c	52	0.01	0	0
623	21	16	3 <sup>h</sup>	m	13	0.35	85	0.30
627	35	2	3 <sup>h</sup> 10'	m	63	0.18	35	0.06
628	29	—	2 <sup>h</sup> 55'	c	41	0.44	22	0.10
640	—	8	3 <sup>h</sup> 15'	m	56	0.05	28	0.01
641	26	7	2 <sup>h</sup> 45'	c	25	0.33	40	0.13
648	35	6	3 <sup>h</sup> 05'	m	32	0.60	35	0.21
649	43	5	4 <sup>h</sup> 15'	c	24	0.83	91	0.76
650	43	7	2 <sup>h</sup> 45'	c	12	0.49	84	0.40
664	34	7	4 <sup>h</sup>	c	15	1.39	84	1.15
665	30	6	2 <sup>h</sup> 30'	c	36	1.32	85	1.12
668	45	6	2 <sup>h</sup> 10'	c	17	0.31	47	0.15
669	25	6	2 <sup>h</sup> 15'	m	28	0.36	40	0.15
670	38	8	1 <sup>h</sup> 50'	m	36	0.87	10	0.09
684	26	5	4 <sup>h</sup> 15'	c	35	1.04	84	0.87
696	29	5	2 <sup>h</sup> 35'	m	66	0.19	75	0.15
706	30	5	2 <sup>h</sup> 40'	c	55	0.02	28	0.01
701	27	5	2 <sup>h</sup> 25'	c	44	0.47	72	0.34
703	24	8	2 <sup>h</sup> 45'	c	21	0.75	91	0.68
706	—	8	3 <sup>h</sup> 50'	c	52	0.11	2	0
708	31	8	2 <sup>h</sup> 30'	c	29	0.11	59	0.07
723	39	6	1 <sup>h</sup> 45'	c	53	0.31	77	0.24
728	26	7	2 <sup>h</sup>	c	63	0.54	71	0.38
730	30	4	2 <sup>h</sup> 30'	m	—	0.02	33	0.01
737	39	8	2 <sup>h</sup> 15'	m	20	1.09	89	0.97
749	24	5	3 <sup>h</sup>	c	23	1.01	77	0.78
750	30	5	1 <sup>h</sup> 35'	m	64	0.03	63	0.02
768	50	25	2 <sup>h</sup> 20'	c	35	1.75	71	1.24
782	—	4	1 <sup>h</sup> 50'	m	45	0.17	46	0.08
784	22	5	2 <sup>h</sup> 05'	m	62	0.04	0	0
794	32	5	2 <sup>h</sup> 40'	c	29	1.10	85	0.94

## CASES OF AZOOSPERMIA

469	38	4	2 <sup>h</sup> 15'	m
553	36	11	3 <sup>h</sup> 45'	c
637	27	6	4 <sup>h</sup> 10'	c
686	29	6	3 <sup>h</sup> 07'	c





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